Characterization of J822 —

a novel cold induced GTP-binding protein gene in wheat (Triticum aestivum)

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ABSTRACT

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Cold tolerance in plants is a multigenic trait and high levels of tolerance require a period of acclimation during which the expression levels of many genes are known to change. The study of the signaling and regulatory mechanisms that control acclimation is particularly important for understanding the genetic basis of cold tolerance in plants. Previous microarray analysis has identified a number of genes that have altered expression during cold acclimation, some of which have characteristics of signaling or regulatory proteins. Since GTP-binding proteins have been shown to be important regulatory proteins in animal systems, the recently discovered cold regulated GTP-binding-like protein, J822, from wheat, was chosen for this study.

The predicted amino acid sequence of *J822* indicated that it encodes a novel GTP-binding protein. Cold induction of transcript level of *J822* is higher in cold tolerant winter cultivars than in spring wheat cultivars. Yeast two-hybrid screening showed that J822 interacts with two phospholipase C proteins. J822 and the phospholipase Cs were found to be regulated by cold, drought and salt stress and may be involved in stress-induced phospholipid signaling. The Arabidopsis homolog of *J822* is also regulated by cold, and provides a promising avenue for the functional study of this GTP binding protein as part of the stress response.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABA:	Abscisic Acid
ABRC:	Arabidopsis Biological Resource Center
ABRE:	Abscisic acid responsive element
AOS:	Active Oxygen Species
BLAST:	Basic Local Alignment Search Tool
CBF:	CRT/DRE Binding Factor
CDPK:	Ca^{2+}-Dependent Protein Kinases
COR genes:	Cold Regulated genes
CRT:	C-Repeat
DAG:	diacylglycerol
DGK:	diacylglycerol Kinase
DRE:	Drought Responsive Element
DREB:	DRE Binding proteins
EBI:	European Bioinformatics Institute
EF-1 α:	elongation factor-1 alpha
ER:	Endoplasmic Reticulum
EST:	Expressed Sequence Tag
FGAS:	Functional Genomics of Abiotic Stress
GST:	glutathione *S*-transferase
Hik:	Histidine Kinase
ICE :	Inducer of CBF Expression

IP_3:	inositol 1,4,5-triphosphate
LB:	Luria-Bertani
LB:	Left Border primer
LEA:	Late Embryogenesis Abundant
LT:	Low Temperature
MAPK:	Mitogen-Activated Protein Kinase
NCBI:	National Center for Biotechnology Information
NO:	Nitric Oxide
ORF:	Open Reading Frame
PA:	Phosphatidic Acid
PAK:	Phosphatidic Acid Kinase
PC:	phosphatidylcholine
PC-PLC:	phosphatidylcholine-specific phospholipase C
PCR:	Polymerase Chain Reaction
PE:	phophatidylethanolamine
PG-PLC:	phosphatidylglycerol specific phospholipase C
P*I*:	isoelectric point
PI-PLC:	phosphoinositide-specific phospholipase C
PIP_2:	phosphatidylinositol 4,5-bisphosphate
PLC:	phospholipase C
PLD:	phospholipase D
PtdIn:	phosphatidylinositol
QTL:	Quantitative Trait Loci

ROS:	Reactive Oxygen Species
RT:	Reverse Transcriptase
SAIL:	Syngenta Arabidopsis Insertion Library
SIGnAL:	Salk Institute Genomic Analysis Laboratory
SNP:	sodium nitroprusside
TAIR:	The Arabidopsis Information Resource
TIGR:	The Institute of Genomic Research
UTR:	untranslated region

PART I. INTRODUCTION

The major abiotic stresses encountered by plants are drought, cold, heat, salinity, soil mineral deficiency and soil mineral toxicity. Unlike animals, plants are immobile and can not avoid unfavorable environments. For survival, plants have evolved adaptive mechanisms that permit plant cells to sense outside stimuli and make coordinated physiological and structural adjustments to enhance tolerance for different environmental stresses. The mechanisms of plant responses to environmental stresses are complex and include genetic, biochemical, physiological and even morphological changes. Plant environmental stress tolerance is very important to agriculture. Crop production is hardly ever free of environmental stress, and there are massive annual crop yield losses associated with stress. Research focused at understanding plant environmental stress tolerance mechanisms has potential practical applications that can benefit agriculture throughout the world.

Low temperature (LT) stress includes freezing and chilling, both of which can cause plant injury, since the range of temperature over which plants are damaged by low temperature conditions vary widely among different species. A number of species, notably temperate cereals including wheat, barley, oats and rye, can survive temperatures of -15°C or lower if the plants are fully acclimated. Rye, the most cold tolerant cereal, can survive temperatures as low as -30°C. Many other important crops, such as cotton, soybean, rice, tomato, and maize, are chilling sensitive and can be damaged at + 4°C and in the case of some tropical fruits damage can occur at +12 °C (Lyons and Raison, 1970). In addition to crop loss, LT affects the quality of the products and the storage

ability of fruits or vegetables (Graham and Patterson, 1982). Extensive understanding of the molecular basis of low temperature tolerance mechanism is essential to development of new approaches to improve plant LT tolerance, to be able to increase crop yield and to expand lands available to agriculture.

1. PLANT LOW TEMPERATURE INJURY

The symptoms of LT injury include surface lesions, water-soaked appearance of the tissues, water loss or desiccation, internal discoloration, tissue breakdown, accelerated senescence, shortened shelf life and faster decay due to leakage of plant metabolites. Physiological damage is classified as primary injury and secondary injury based on the effects and intensity of LT stress. Primary injury is the initial rapid response to the LT stress that causes dysfunction in the plant and is reversible if the conditions return to physiological temperatures. Secondary injury is a consequence of primary injury and causes irreversible damage to the plasma membrane and the metabolic function of the plant cell. LT causes three main problems in plant cells: changes in the spatial organization of cellular membranes, retardation of biochemical and chemical reactions and alterations in the availability of water (Vézina et al., 1997). As temperatures fall below 0°C, ice forms in the intracellular or extracellular spaces of plant tissues. The most common form of freezing in plant tissues occurs in the extracellular space within the tissue of plant. In susceptible plants intracellular freezing occurs and causes cell and tissue death (Burke et al., 1976).

One source of freezing injury is cellular dehydration. The chemical potential of ice is less than that of liquid water. Ice formation between cells causes a movement of

unfrozen water from inside the cell to the extracellular space (Levitt, 1980; Steponkus and Webb, 1992). Freezing induced dehydration causes serious damage to membranes along with other deleterious effects. Freezing induced dehydration causes lipid associations to change from lamella to hexagonal-II structures (Steponkus et al., 1993). Freezing temperatures also cause protein denaturation (Guy et al., 1998). There is evidence that freezing temperature can trigger the production of reactive oxygen species, that can cause cellular rupture by inducing adhesions between extracellular ice and cell wall or cell membrane (McKersie and Bowley, 1997).

2. FREEZING TOLERANCE MECHANISMS AND COLD ACCLIMATION

Plants vary greatly in their ability to survive freezing temperatures. There is a wide range of freezing tolerance both between and within species. Some plants, such as potato (*Solanum tuberosum*) cannot withstand any freezing, whereas cereals such as wheat, barley and rye can survive below freezing temperature during a long winter season. There can be considerable variation in freezing tolerance within the same species. For example, winter cultivars of wheat and barley are more cold tolerant than spring cultivars (Fowler et al., 1999). The most tolerant species require acclimation in order to achieve their highest degree of LT tolerance. During cold acclimation, plants initiate mechanisms to prevent or survive freezing injury, and an essential part of cold tolerance is the ability to recover from freeze-induced injury (Howarth & Ougham 1993). For example, nonacclimated rye is killed at about -5°C, but after cold acclimation at 4°C for 3 weeks, it can survive freezing at -30°C (Thomashow, 1999). In addition to low-non

freezing temperature, cold acclimation can be triggered by drought stress and exogenous application of abscisic acid (ABA) (Lang et al., 1989; Guy et al., 1992; Mäntylä et al., 1995).

Studies have shown that the mechanism of plant cold tolerance is complex and involves a number of interactive signal pathways as that effect the expression of many genes (Thomashow, 1999; Zhu, 2001). It is a major challenge to identify all of the components involved in mechanism of cold tolerance and to unravel the intracellular communication that regulates them. However, one key factor that has been clearly demonstrated is that cold acclimation stabilizes and protects cell membrane against freezing damage.

2.1. Cold acclimation prevents formation of hexagonal-II phase lipids and cell lysis

Steponkus and colleagues (1993) reported that cycles of osmotic contraction and expansion of non-acclimated winter rye plants occurred with freezing-thawing cycling at temperature down to -2°C and -4 °C, and that freeze-thaw stress induced cell lysis. At temperatures between -4 and -10 °C, non-acclimated plant membranes have freeze induced transition from lamella to hexagonal-II phase lipid structure, which causes the fusion of cellular membranes. Cold acclimated plants did not suffer this kind of injury. The hexagonal- II phase was not observed in leaves of cold-acclimated rye when frozen to -35 °C (Webb and Steponkus, 1993).

2.2. Cold acclimation results in alteration of lipid composition

Unsaturated phospholipids have lower freezing temperature than saturated phospholipids. Lynch and Steponkus (1987) reported that total phospholipids in plant membranes increased significantly during cold acclimation, and di-unsaturated phospholipids made up a substantial part of this change. An increase in the degree of membrane lipid unsaturation, with accompanying increase in bilayer fluidity is an important determinant of cold acclimation. This study also demonstrated that there are not unique lipid species in membranes isolated from non-acclimated and cold acclimation plants. Alteration in lipid composition results in different behavior of the plasma membrane. Plasma membranes isolated from noacclimation plants undergo endocytotic vesiculation, with a large surface reduction as a result of expansion-induced lysis. In contrast, plasma membranes from cold acclimation plants form exocytotic extrusions, and the surface area is conserved such that expansion-induced lysis doesn't occur (Dowgert and Steponkus, 1984; Gordon-Kamm and Steponkus, 1984).

2.3. Accumulation of sucrose and other small molecules during cold acclimation

Cold acclimation is associated with physiological and biochemical alterations, that includes the accumulation of sucrose, other simple sugars, amino acids, organic acids, compatible solutes and cryoprotective proteins. The accumulation of some small molecules contributes osmotically to the intracellular hypertonic environment, which prevents freeze-induced dehydration. There are a number of reports on the enhancement of freezing tolerance in plants that is associated with increased metabolic activity, such as

the synthesis of trehalose or glycine betaine (Holmström et al., 1996). For example, transgenic tobacco engineered to produce glycine betaine had improved tolerance to low temperatures (Holmström et al., 2000). Some amino acids, such as free proline, have been shown to be cryoprotectants, and Xin and Browse (1998) has reported that the *esk1* mutant in Arabidopsis plants that has enhance freezing tolerance had 30-fold higher free proline levels than wild type plants. Sucrose and other simple sugars that accumulate during cold acclimation seem likely to contribute to stabilization of membranes, since it was demonstrated that sugars can protect membranes against freezing induced damage in vitro (Anchordoguy et al., 1987).

3. COLD-REGULATED FUNCTIONAL GENES

In 1985, Guy et al. demonstrated that cold acclimation triggers changes in gene expression. Subsequently, a number of cold-induced genes have been isolated and characterized from different plants. Many proteins encoded by cold induced genes have their probable function elucidated from DNA sequence homology. They include antifreeze proteins, lipid transfer proteins, fatty acid desaturases, molecular chaperones, enzymes involved in biosynthesis of cryoprotectants, and proteins involved in signal transduction. There is also a large group of cold induced genes that encode hydrophilic or hydrophobic polypeptides and homologs of late embryogenesis abundant (LEA) proteins with unknown activities, but which have been demonstrated to contribute to freezing tolerance (for review see Thomashow, 1999). Never-the-less, many LT induced gene products still have no predicted function.

From 1985 to 1999, there were about 25 cold regulated genes described with demonstrated biochemical functions or putative activities in Arabidopsis and other plant species. (for detail see Supplemental Material, Thomashow, 1999). In the last few years, advanced technologies such as microarray and mutational analysis and other genomic-scale approaches were widely applied to isolation of cold-induced or freezing tolerance genes, these approached have greatly accelerated the pace of gene discovery.

Llorente *et al* (2002) reported that Arabidopsis transgenic plants overexpressing *rci3,* a cold-induced gene encoding a peroxidase, have increased tolerance to dehydration and salt stress. Suppression of RCI3 expression results in dehydration and salt-sensitive phenotypes. Accumulation of reactive oxygen species such as hydrogen peroxide (H_2O_2) can induce the expression of detoxification and stress related genes (Kovtun et al., 2000). *Scgst1* in *Solanum commersonii* encodes a glutathione *S*-transferase (GST), which is regulated by low temperature. Seppanen et al (2000) demonstrated *Scgst1* is strongly induced in the freezing tolerant species *Solanum commersonii*, but the freezing sensitive species *Solanum tuberosum* did not express the same gene. They also demonstrated that active oxygen species (AOS) were associated with the early steps of *Scgst1* regulation.

Osisap1 in rice, a gene encoding a zinc-finger protein, is induced not only by cold stress, but also by desiccation, salt, submergence, and heavy metals. Overexpression of the gene in transgenic tobacco conferred tolerance to cold, dehydration, and salt stress at both the seed-germination and seedling stage (Mukhopadhyay et al., 2004).

4. LOW TEMPERATURE INDUCED SIGNAL TRANSDUCTION

During cold acclimation, perception of low temperature initiates the cold related signaling at the cellular level that results in increased tolerance. Signal transduction is a complex process that involves low temperature sensors, low temperature signal transducers and low temperature induced genes expression. The genes involved are generally classified into two groups: genes that encode products that directly protect plant cells against cold stress, and genes that regulate the expression of the response genes. The later include such classes of genes as transcription factors, protein kinases and phosphatases, RNA binding proteins, and proteins that regulate the degradation of proteins. Elucidation of the signal pathways and components controlling the cold response regulons is one of the key areas in plant cold tolerance research.

4.1. Cold perception in plants

The plasma membrane may be the primary sensor that perceives the cold signal. From studies with *Synechocystis* sp., Vigh et al (1993) proposed that rigidification of the plasma membrane might be the event that initiates the downstream signaling cascade. Cold perception causes actin filament reorganization, that opens Ca^{2+} channels in plasma membrane by loose-tension forces. The physical alteration contributes to Ca^{2+} influx in the cytoplasm (Orvar et al., 2000). Secondary low temperature sensors in plants are proposed to include histidine kinases, phospholipases, receptor-like kinases and calcium sensors, which are all located in plasma membrane (Sharma et al, 2005).

4.2. Ca^{2+} function in plant cells, cold tolerance and Ca^{2+} sensors

After the initial cold signal perception, secondary messengers are produced. Commonly known secondary messengers include Ca^{2+}, CaM, cAMP, cGMP, cADP-ribose, inositol 1,4,5-triphosphate (IP_3) and reactive oxygen species (ROS). Secondary messengers initiate a signaling cascade mediated by protein kinases or protein phosphatases (Sharma et al, 2005). Ca^{2+} has been widely implicated as an intracellular messenger of physiologically and environmentally induced signaling pathways in plants (Trewavas and Malho, 1998). Upon environmental stress, a plant cell has a rapid elevation of cellular Ca^{2+}. Increase in intracellular Ca^{2+} has been observed within 10 seconds after cold treatment (Henriksson and Trewavas, 2003). Because cellular Ca^{2+} levels are tightly regulated, small changes in intracellular Ca^{2+} can provide information for the modification of enzyme activity and gene expression. A key step in understanding how Ca^{2+} functions in plant cells is to identify proteins that act as Ca^{2+} sensors. These are proteins that bind Ca^{2+} and change their conformation and function.

In Arabidopsis, the *cbl1* gene, which encodes the CBL1 protein, is a member of the calcineurin-B like protein family. As a calcium binding protein, CBL1 functions as a negative regulator of cold response and positive regulator of salt and drought response in plants. Cheong et al. (2003) reported that *cbl1* null mutant plants displayed enhanced tolerance to freezing and decreased tolerance to salt and drought. In addition, transgenic plants overexpressing CBL1 showed reduced tolerance to freezing and enhanced tolerance to salt and drought.

Ca^{2+}-dependent protein kinases (CDPK) are another kind of Ca^{2+} sensor. They are a common transduction component that interpret Ca^{2+} signals in plant cell and control cellular metabolism (Cheng et al., 2002). Several CDPK proteins that regulate freezing or other environmental stress tolerances in plants have been identified. A rice CDPK gene, *Oscdpk7*, has been shown to be induced by cold and salt stress. Transgenic rice plants overexpressing OsCDPK7 were shown to have enhanced tolerance to cold and salt. Interestingly, over-expression of OsCDPK7 only enhanced some salt stress-responsive genes induction, and did not induce cold-stress regulated genes. Thus, it was suggested that the downstream pathways leading to the cold and salt tolerance are different from each other. (Saijo et al., 2000). Sheen (1996) reported that CDPK induced the expression of a stress-responsive gene *hva1* in maize. Protein phosphatase type 2C can block the CDPK activation. Tahtiharju and Palva (2001) generated phosphatase type 2C -silenced Arabidopsis plants and found that there was an enhanced induction of *cbf1*, *rab18*, *rcI2A*, and *ltI78* expression in the silenced lines under cold or ABA treatment, and the transgenic plants exhibited a higher degree of cold acclimation. These studies suggested that cold-induced CDPKs are involved in the regulation of expression of some cold-responsive genes.

Cytoplasmic Ca^{2+} also plays a role in the regulation of phospholipase D (PLD) in plants. Sequence analysis indicates that plant PLDs contain a Ca^{2+} /phospholipid-binding fold, called the C2 domain, at the N terminus (Pappan et al., 1997). This domain is involved in Ca^{2+} regulated membrane association and protein-protein interactions. The C2 domains of PLD α and β have been demonstrated to bind Ca^{2+}, and this binding causes conformational changes of the proteins (Zheng et al., 2000).

4.3. Reactive oxygen species (ROS) enhance cold and other stress tolerances in plants

Cold, drought and salt stresses all induce the accumulation of ROS, such as hydrogen peroxide, superoxide and hydroxyl radicals (Hasegawa et al., 2000). These ROS can be damaging agents, which contribute to stress injury in plants, and may also act as signals involved in the induction of ROS scavengers and other protective mechanisms (Prasad et al., 1994). Transgenic plants which overexpress ROS scavengers or mutants with high scavenging ability show increased tolerance to environmental stresses (for review see Bohnert and Sheveleva, 1998). Some genes related to abiotic stress signaling, such as the transcription factor DREB2A or Hik, have been shown to be upregulated by oxidative stress (Desikan et al., 2001). AtMAPK6 in Arabidopsis can be activated by both cold stress and oxidative stress (Yuasa et al., 2001). All the evidence suggests that ROS are involved in cold-induced signaling as secondary messengers.

4.4. Histidine kinases (Hik) mediate membrane fluidity changes and activate the mitogen-activated protein kinase (MAPK) pathway

A two-component histidine kinase (Hik) acts as a membrane protein sensor for low temperature perception, perhaps by the detection of changes in membrane fluidity during cold acclimation. Suzuki et al (2000) reported that Hik33, Hik19 and a response regulator (Rer1) in *Synechocystis* are involved in cold induced signaling. When plants encounter cold temperature or high concentration of saturated fatty acids, the histidine residue in Hik33 kinase domain is phosphorylated, and transfers a phosphate group to

Hik19, which, in turn, is transferred to Rer 1. This phosphorylation pathway regulates expression of the desaturase gene, *DesB*, which increases the concentration of unsaturated fatty acids in membrane lipid.

Cold stress stimulates the accumulation of compatible osmolytes and antioxidants in plants (Hasegawa et al., 2000). In Arabidopsis, histidine kinases have been identified, as receptor type proteins that activate the MAPK pathway, which is responsible for the production of compatible osmolytes and antioxidants (Urao et al., 1999).

4.5. Phospholipid signaling in animals

Inositol triphosphate (IP_3) is a well-studied signal molecule in animal cells. In mammals, the binding of many hormones to cell-surface receptors induces the release of Ca^{2+} into the cytosol from the endoplasmic reticulum (ER) or other intracellular vesicles. The mechanism of hormone-receptor signal transduction from the cell-surface to ER became clear in the early 1980s. The increase in cytosolic Ca^{2+} is often preceded by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid found in plasma membrane. The binding of hormones to their receptor triggers activation of specific G proteins, which activates phospholipase C (PLC) which hydrolyse PIP_2 to diacylglycerol (DAG) and IP_3. DAG remains in the membrane and functions to activate protein kinase C, which regulates downstream gene expression. IP_3 is water soluble and diffuses to the ER surface, where it binds to a specific IP_3 receptor. A Ca^{2+} channel composed of four subunits, each containing an IP_3 -binding site, is induced to open by IP_3 allowing Ca^{2+} to exit from ER into the cytosol. IP_3 is further converted by the actions of

several distinct kinases and phosphatases to a variety of inositol phosphates, some of which are also implicated in intracellular signaling (Berridge, 1993; Birnbaumer, 1992).

4.6. Phospholipases in Plants

Phospholipases are a diverse set of enzymes that hydrolyze phospholipids. Multiple forms of phospholipase D, C, and A have been characterized in plants. These enzymes are involved in a broad range of functions in cellular regulation, lipid metabolism, and membrane remodelling. In recent years, increasing attention has been paid to the many roles of phospholipases C and D in signal transduction. Vergnolle et al.(2005) recently analyzed the transcriptome of cold treated Arabidopsis suspension cells in presence of inhibitors of PLCs and PLDs and reported that the expression of most cold responsive genes were affected by the inhibitors. Their results suggest that both the PLCs and PLDs are elements of two distinct cold signaling pathways.

Phospholipases are classified into five major groups: phospholipase D (PLD), C (PLC), A2 (PLA2), A1 (PLA1), and B (PLB) according to their sites of hydrolysis on phospholipids (shown in Figure 1). Each class is further divided into subfamilies based on their amino acid sequence, biochemical properties and their substrate specificity. There are two sub groups of PLC: the better-characterized phosphoinositide-specific phospholipase C (PI-PLC), that hydrolyses PIP_2 (phosphatidylinositol 4, 5-bisphosphate), and the phosphatidylcholine-specific phospholipase C (PC-PLCs) that hydrolyse the common membrane phospholipid, PC, as well as some other phospholipids (Wang et al., 2002). Phospholipase D has three subclasses based on the requirement for Ca^{2+} and lipid specificity: (1) the conventional PLD that is most active at millimolar levels of Ca^{2+} (20

to 100 mM), (2) the polyphosphoinositide (PI)-dependent PLD that is most active at micromolar levels of Ca^{2+}, and (3) the phosphatidylinositol (PtdIn)-specific PLD that is Ca^{2+} -independent (Wang et al., 2002).

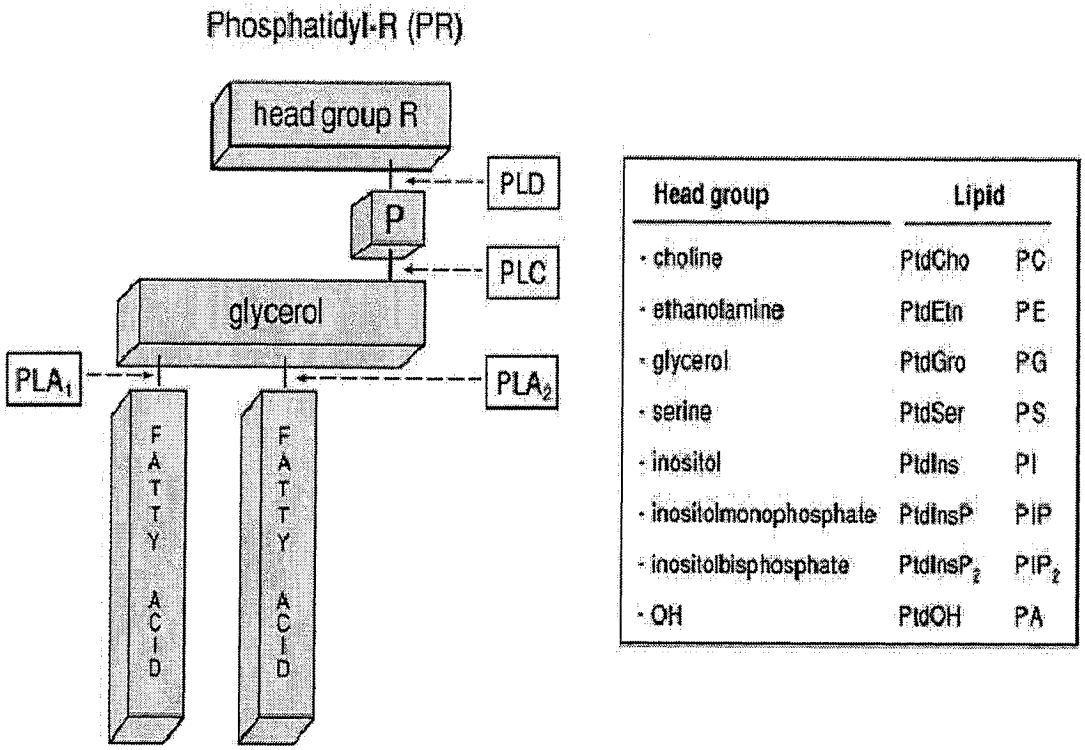

Figure 1. Common phospholipids structure and phospholipase hydrolysis sites. The general structure of a phospholipid consists of two fatty acyl chains esterified to a glycerol backbone at the *sn*-1 and -2 positions, a phosphate at the *sn*-3 position (creating the "phosphatidyl" moiety), to which a variable head group (R) is attached. The positions that are subject to phospholipase activity are indicated.
Head groups are listed in the box together with the common abbreviations. (The figure is taken from Meijer and Munnik, 2003)

4.7. Phospholipid signaling in plants

Since De Nisi and Zocchi (1996) reported that cold treatment induces changes in the level of membrane polyphosphoinositide in roots of maize seedlings, and Knight et al. (1996) showed the increase in cytoplasmic calcium could be the result of IP_3 metabolism in Arabidopsis, scientists have proposed that some membrane enzymes, such as PLC and PLD, are involved in the cold signal transduction pathways. Although phospholipid signaling has not been widely studied in plants, it has been shown that IP_3, IP_6 and PA act as messengers in plants, whereas only IP_3 and DAG are known to act as signals in animals (Figure 2). Phospholipid signaling is an important component in signal transduction pathways and plays a key role in response to environmental stresses in plants. Lipid derived signals can activate enzymes or recruit proteins to membranes, where the local increase in concentration promotes interactions and downstream signaling. There are many reports that hyperosmotic stress or drought activates phospholipase C signaling (Munnik and Meijer, 2001).

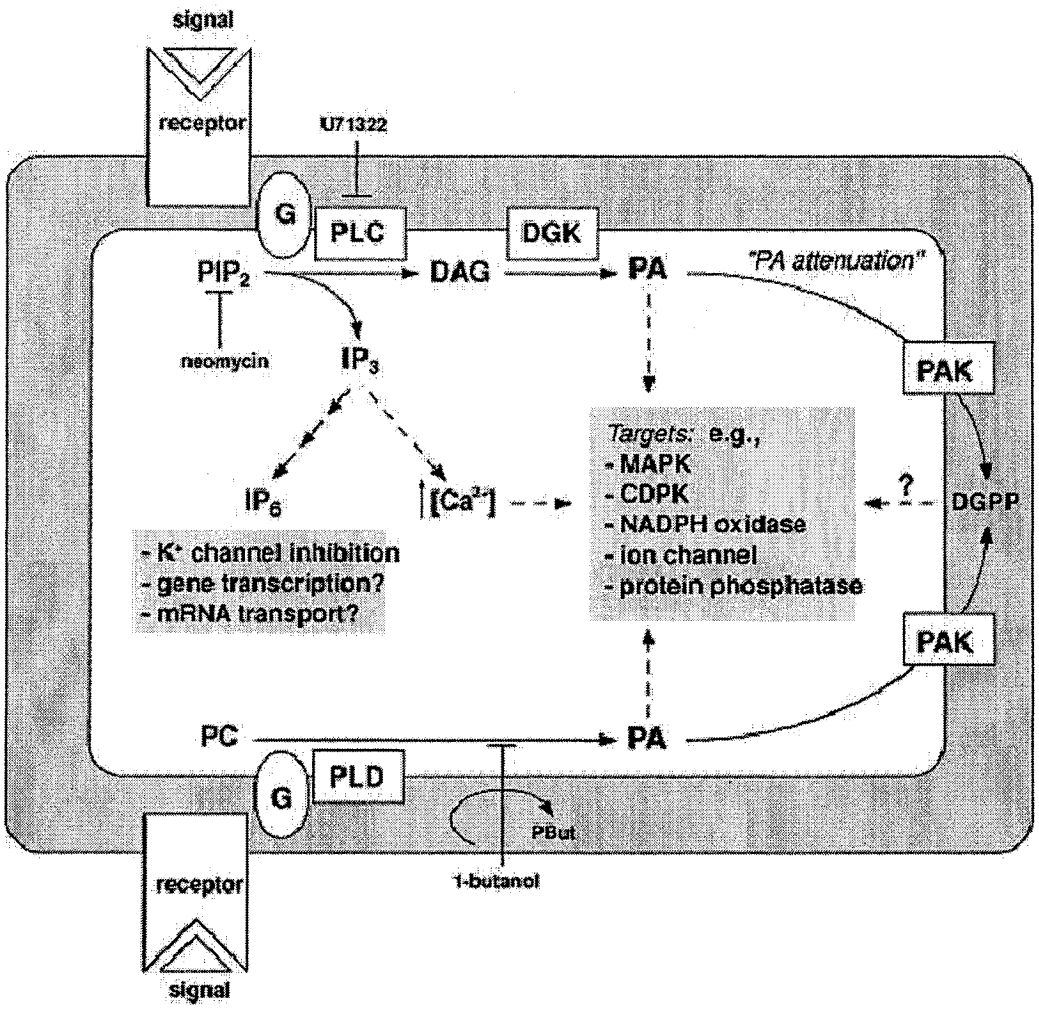

Figure 2. Model of PLC and PLD signaling in plants.

PLC hydrolyzes PIP_2 into IP_3 and DAG. IP_3 diffuses into the cytosol where it triggers the release of Ca^{2+} from intracellular stores or it is converted to IP_6, triggering nuclear responses. DAG remains in the membrane where it is phosphorylated to PA by DAG kinase (DGK).

PLD generates PA directly by hydrolyzing structural lipids such as phosphatidylcholine (PC). Increased PA levels affect several plant processes via various intracellular targets. Signaling is attenuated when PA is phosphorylated to diacylglycerol pyrophosphate (DGPP) by PA kinase (PAK). Because DGPP is a minor lipid that dramatically increases in concentration when cells are activated, it could itself be a signaling molecule. Inhibitors and the putative involvement of G-protein (G)–coupled receptors are shown. (Figure is taken from Meijer and Munnik, 2003)

4.7.1. IP_3 serves as second messenger

Cellular plasma membrane-bound PLC and PLD contribute to intracellular Ca^{2+} increases during cold acclimation. PLC and PLD are thought to act as low temperature secondary sensors, and to cause increases in cytosolic IP_3 and DAG in response to low temperatures (Teun, 2001; Munnik, 1998; Ruelland et al., 2002). Alexandre et al (1990) demonstrated, that IP_3 serves as second messenger to mediate Ca^{2+} release from the tonoplast. IP_3 is rapidly induced in red beet by 0 °C treatment to levels ten-fold higher than plants treated at 10 °C (Ruelland et al., 2002). IP_3 has also been shown to act in ABA-mediated stress responses such as the regulation of stomatal apertures in tobacco guard cells (Hunt et al., 2003). When treated with ABA, guard cells of *Solanum tuberosum* and *Vicia faba* increase IP_6 levels (Lemtiri-Chlieh et al., 2000), and osmotically stressed *Schizosaccharomyces pombe* rapidly metabolized IP_3 to IP_6 (Ongusaha et al., 1998).

4.7.2. Phosphatidic acid (PA) and PIP_2

Phosphatidic acid can be generated by two different phospholipid-signaling pathways. PLD produces PA directly by hydrolysing structural phospholipids, such as phosphatidylcholine (PC) and phophatidylethanolamine (PE). PLC generates PA indirectly by the hydrolysis of PIP_2 to generate IP_3 and DAG, and DAG is immediately phosphorylated to produce PA by DAG kinase (DGK) (Meijer and Munnik, 2003; Munnik, 2001). Though DAG is known to act as a second messenger in animals, this has not been demonstrated in plants. As a substrate of PLC and PLD, PIP_2 itself is a signal molecule and may be involved in several processes, such as the recruitment of signaling

complexes to specific membrane locations (Martin, 1998). During osmotic stress, plant cells increase the production of PIP_2 by inducing the expression of *PI5K*, a gene that encodes a phosphatidylinositol 4-phosphate 5-kinase which is involved in the synthesis of PIP_2 (Mikami et al., 1998). Consistent with this observation, osmotic stress was found to rapidly increase PIP_2 levels in cultured Arabidopsis cells (Pical et al., 1999; De Wald et al., 2001)

Increases in PA have been observed in many plant cell types in response to osmotic stress, wounding, ABA, pathogen attack, oxidative stress and drought stress (Munnik, 2001). The accumulation of PA occurs within minutes after plant tissues have been subjected to the stress. Ruelland et al (2002) reported that PA formation is induced by cold treatment and that about 80% of the cold-induced PA comes from PLC hydrolysis and the other 20% comes from the activity of PLD. Several downstream targets of PA have been identified, including protein kinases and ion channels (Munnik, 2001), and the inhibition of PA synthesis has been shown to impede downstream responses. In Arabidopsis, the suppression of PLD expression results in a decrease in osmotic stress related responses (Wang et al., 2000). Blocking PLC enzyme activity inhibits activation of several defence responses, including the formation of ROS (De Jong et al., 2004). In addition, Jacob et al (1999) reported that PA mimics ABA actions in plant guard cells.

4.7.3. Relationship between GTP-binding proteins and PLCs

The mechanism by which extracellular information is received, transduced and converted into intracellular response in plants is not fully understood. In the animal

model of PLC, signaling is initiated by ligand-receptors which activate G proteins, which activate intracellular effector enzymes such as PLC and PLD to produce second messengers (IP_3, DAG or PA). The existence of homologous genes for these proteins suggests that similar signaling also functions in plants. However the successive interaction and activation of the proteins has not yet been demonstrated in plants and the description of the pathways remains uncertain. Biochemical and molecular analyses have demonstrated that heterotrimeric G proteins are present in plants (reviewed by Ma, 1994), and their activation has been shown to stimulate effector enzymes and induce physiological responses (Bolwell et al., 1991). Some G proteins have been identified in plants to be involved in stress regulation. G-protein–associated receptors might also serve as membrane-bound receptors for regulating ion channels and ABA signaling in Arabidopsis guard cells. In Arabidopsis, the *gpa1* gene encodes α subunit of a G protein. Mutants of *gpa1* have a disrupted stomatal regulation, are insensitive to ABA, and the rate of water loss from *gpa1* mutants is greater than that from wild-type plants (Wang et al., 2001).

The best evidence by which G protein have been demonstrated to activate PLC in plants is provided by Apone et al (2003). They showed that enhanced thymidine incorporation into DNA by a G protein-coupled receptor is dependant on an increase in PI-PLC activity and an elevation of inositol 1, 4, 5-trisphosphate levels in *Nicotiana tabacum* cells. Other studies used mastoparan, a G protein activator, which affects G protein GTP/GDP state in favour of its activated GTP form. Mastoparan activates heterotrimeric G proteins by a mechanism that mimics receptor activation. This makes it a valuable tool for testing whether effector enzymes, such as PLC or PLD, are activated

via G proteins (Law and Northrop, 1994; Ross and Higashijima, 1994). Legendre et al (1993) reported mastoparan stimulates phospholipase C activity and leads to an increase in IP_3 in suspension-cultured soybean cells. Cho et al. (1995) found that mastoparan stimulated PI-PLC activity without permeabilizing *Chlamydomonas* cells. Van Himbergen et al (1999) reported that the level of mastoparan required for activating PI-PLC was ten-fold higher than that for activating PLD. Based on the study of mastoparan in maize root cells, Baluska et al. (2001) suggested that mastoparan-mediated activation of heterotrimeric G-proteins induced relocalization of profilin from nuclei into the cytoplasm of root apex cells. Presumably the G protein acts by triggering a phosphoinositide-signaling pathway based on phosphatidylinositol-4, 5-bisphosphate (PIP_2). Although our understanding of the roles of G proteins in plant signal transduction is still in their infancy, these studies indicate that there is a strong relationship between G proteins and phospholipase C.

4.8. ABA and signaling for genes with DRE/CRT or ABRE *cis*-elements

Plant hormones are important regulators of plant growth and development, as well as regulating the response to environmental signals. ABA has been implicated in the response to a number of biotic and abiotic stresses. Though the ABA levels increased in response to stresses, the relationship between ABA and different stress signaling pathways is not completely understood. The general conclusion is that low-temperature regulated gene expression is largely independent of ABA, and that osmotic stress regulated genes can be activated through both ABA-dependent and ABA-independent pathways (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

Gene expression profiling using microarrays has identified many genes that are regulated by cold, drought or other stress. Although it is almost impossible to determine regulatory mechanisms for every gene, stress responsive genes with DRE/CRT or ABRE *cis*-elements serve as an excellent model for understanding such signaling. Several stress regulated genes have combinations of these cis elements in their promoters (Loake et al., 1992). Cold, drought and salt stress can activate genes through cold-inducible transcription factors CBF/DREB1. DREB2 has been identified as an osmotic stress-inducible transcription factors and ABF/AREB are ABA-inducible transcription factors (Xiong et al., 2002). A transcriptional activator, ICE, that acts upstream of CBF in the cold signal pathway, may be ABA independent. ICE is present in non-stressed plants in an inactivated state, is activated by a cold induced signal, and become an inducer of CBF expression (Chinnusamy et al., 2003). Hos1 negatively regulates cold signaling, presumably by targeting ICE or upstream signaling components for degradation (Lee et al., 2001). *Arabidopsis* COR genes play an important role in cold acclimation (Jaglo-Ottosen et al., 1998). The promoters of these genes contain C-repeat (CRT) or dehydration responsive elements (DRE), which are found in many stress responsive genes and are sufficient to impart stress responsiveness (Yamaguchi-Shinozaki and Shinozaki, 1994). Transcriptional activators from the CBF- family of proteins bind to CRT/DRE sequences and activate the expression of COR genes, which in turn, promote freezing tolerance (Jaglo-Ottosen et al., 1998).

5. CURRENT MAIN APPROACHES FOR RESEARCH ON COLD-REGULATED GENES

Since the completion of the entire Arabidopsis genome sequence (Arabidopsis Genome Initiate, 2000) and the release of a rice genome sequence draft to the public (The International Rice Genome Sequencing Project, 2002), rapid progress is being made in mapping and sequencing the genomes of several important crop species. These data, together with expressed sequence tags (ESTs) from many other species including maize, soybean, and wheat, constitute a wealth of genetic information on important crop species, and provide a rich resource for researchers to identify genes and determine their functions.

The development of microarrays has allowed important progress in last decade. Gene expression profiling has become an important tool to investigate how an organism responds to environmental changes. It has advanced the rate of discovery of genes that are regulated by cold and other stress and especially facilitated the identification of regulatory genes that are stress regulated (Hazen et al., 2003). T-DNA and gene silencing techniques have also been widely applied to the characterization of genes involved in freezing tolerance. In recent years there has been marked increase in the development of mutant stocks, RNA interference and virus induced gene silencing methods (AzpirozLeehan and Feldmann, 1997; Wesley et al., 2001). With the dramatic increase in the number and availability of mutant lines, the functions of many genes have been determined and novel signaling elements have been identified. For example, using RD29A-LUC transgene as a reporter, Ishitani et al. (1997) have identified 103 candidates of stress signaling components from an Arabidopsis T-DNA mutant pool.

In addition, there has been a significant expansion of mapping of quantitative trait loci (QTL) to identify environmental stress-tolerance loci. The yeast two-hybrid system

has been used to elucidate signaling through the detection of protein-protein interaction. These methods have been applied to the study of environmental stress tolerance, and to advance the understanding of cold signaling mechanisms in plants.

6. *J822*, A NOVEL COLD-REGULATED GENE IN WHEAT

J822 is a cold induced gene, which was identified in a previous study of gene expression by microarray analysis (Gulick et al., 2005). Its amino acid sequence indicated that *J822* encodes a GTP-binding protein. The level of cold induction of *J822* is different between winter and spring wheat. Yeast two-hybrid screening showed that J822 interacts with PI-PLC1 and PG-PLC1. The goal of this study is to characterize *J822* and investigate its role in the plants stress response. The characterization of this gene is the subject of the work presented here.

PART II. MATERIALS AND METHODS

Standard techniques in molecular biology for preparation of LB culture media, electrophoresis and solutions for DNA precipitation were prepared and carried out according to Sambrook et al (1989). Plasmid isolation was done with QIAGEN Spin Miniprep Kit (Qiagen). Cloning and subcloning were done by using Zero Blunt® TOPO® PCR cloning Kit (Invitrogen). RNA samples were purified by DNA- free™ (Ambion) and quantified by UV-visible spectrophotometer (Cary 50Bio, VARIAN). Reverse transcription of RNA samples were done with Superscript™ First-strand Synthesis System for RT-PCR (Invitrogen). UV images were taken by GENE GENIUS BIO IMAGING SYSTEM (SYNGENE), and the data analysis of acquired gel images were done with using software Gene Tools (Version 3.00.22) and GeneSnap (version 4.00) provided by the manufacture.

1. *J822* FULL-LENGTH cDNA CLONING

J822 was identified as a cold acclimation induced gene in previous cDNA microarray results (Gulick et al., 2005). The original clone was a partial length cDNA clone which was missing approximately 200 bp from the 5' end. An EST sequence (GI:25546899) was identified in the wheat TIGR gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=wheat), which was 97% identical to *J822* cDNA , was 242 bp longer at the 5' end and included a start codon. The EST sequence was used to design a 5' oligonucloetide primer corresponding to the 5'UTR of the gene, and the *J822* sequence was used to design a primer for the 3' UTR (the corresponding gene-specific primers P_1A and P_1B, Table 1). Five cDNA libraries made from *T. aestivum*, referred

to as L2, L3, L4, L5 and L6 were made available to us by Dr. Jean Danyluk, from Université du Québec à Montréal (UQAM). Polymerase Chain Reaction (PCR) amplification was carried out with plasmid from each cDNA library by high fidelity polymerase enzyme Pfu (Fermentas products, 2.5 Units/ul). PCR temperature cycling used was: 94°C for 2 minutes, followed by 36 cycles of 45 seconds at 94°C, 45 seconds at 58°C, 3 minutes at 72°C; this was followed by 5 minutes at 72°C. The PCR products were then held at 4 °C. Aliquots of the reaction were electrophoresed in 1 % Agarose gel with Ethedium Bromide (1: 20,000) to detect the PCR products. The size of the PCR fragments was measured by comparison to 1 Kb DNA standard ladder (Fermentas, 0.5 ug/uL). The PCR products from L3, L5 and L6 were respectively subcloned into TOPO vector and subsequently transformed into the TOP10 competent *E. coli* using the protocol of the manufacturer (Zero Blunt® TOPO® PCR cloning Kit, Invitrogen). The transformants were selected on LB media with 50ug/uL Kanamycin. Single colonies were used to inoculate liquid LB media with 50ug/uL Kanamycin which was shaken overnight at 37°C. A pair of *J822* gene specific primers (P_29 and P_7), nested inside of the cloning primers were used to identify 6 positive clones (A7 and A12 from L3, B3 and B11 from L6, C9 and C12 from L5) from culture lysate. Plasmid was purified by using QIAprep® Spin Miniprep Kit (QIAGEN). The 6 plasmids were sent to the Génome Québec Innovation Centre at McGill University for sequencing. All the primers used in this study are listed in the Table 1.

Table 1. PCR primers used in this study

Primer	Sequence(5'-3')	description
P_1A	CCAGCCCAAAAACCCTAGAC	*J822* 5' UTR for cDNA and genomic DNA cloning
P_1B	AACTCAATAAAGGGACCTCAGC	*J822* 3' UTR for cDNA and genomic DNA cloning
P_29	TGGTTTTCATCGGCCATGTTGATG	*J822* inside the cloning primers for positive clone screening, forward
P_7	AGCAGATATGACCAGAACACCAAT	*J822* inside the cloning primers for positive clone screening, reverse
M13-F	GTAAAACGACGGCCAG	Universal forward primer in TOPO vector for *J822* cDNA and genomic DNA sequencing
M13-R	CAGGAAACAGCTATGAC	Universal reverse primer in TOPO vector for *J822* cDNA and genomic DNA sequencing
P_2A	AGCTCCCTCGCTCCCCTC	*J822* 5' UTR for genomic DNA cloning
P_2B	TTCTCCCATCATCCTGTCAAAA	*J822* 3' UTR for genomic DNA cloning
P_3A	CTCTGTTGTCGAGGAGTGTGAG	*J822* last 300 bp in ORF region for partial genomic DNA cloning and RT-PCR, forward
P_3B	GACCTGCCAACTGGAGGAAC	*J822* last 300 bp in ORF region for partial genomic DNA cloning and RT-PCR, reverse
P_5A	GAGGAAATTGACATGAAGAAAGC	*J822* inside the partial genomic DNA cloning primers for positive clones screening, forward
P_5B	CTTGCCTACAGCTATTGTCTTG	*J822* inside the partial genomic DNA cloning primers for positive clones screening, reverse

Name	Sequence(5'-3')	description
P_7A	CTCGCACATTGCTGTCAAGT	*T. aestivum EF-1α* last 300 bp in ORF region for RT-PCR, forward
P_7B	CGGTTGGGTCCTTCTTCTC	*T. aestivum EF-1α* last 300 bp in ORF region for RT-PCR, reverse
UBQ_F	ATGCAGATCTTTGTGAAGACACTCAC	*T. aestivum ubiquitin* first 300 bp in ORF region for RT-PCR internal control, forward
UBQ _R	GTCCTGGATCTTGGCCTTGA	*T. aestivum ubiquitin* first 300 bp in ORF region for RT-PCR internal control, reverse
Tubulin_F	TGAGGTTTGATGGTGCTCTG	*T. aestivum alpha tubulin* gene family conserved region for RT-PCR internal control, forward
Tubulin_R	CCTGGTGGCTGGTAGTTGAT	*T. aestivum alpha tubulin* gene family conserved region for RT-PCR internal control, reverse
PG-PLC_L1	CCCTGCGACAGTAAAGAAGC	*T. aestivum PG-PLC1* last 500 bp in ORF for RT-PCR, forward
PG-PLC_R1	TTGTCACCAGAGCAGATTCG	*T. aestivum PG-PLC1* last 500 bp in ORF for RT-PCR, reverse
PG-PLC_L2	TTCAAGCTCGACGCCTCGC	Homolog of *T. aestivum PG-PLC1* (Cluster 586-contig 2 in FGAS) last 600 bp in ORF for RT-PCR, forward
PG-PLC_R2	GCTCATATCGTGAGTTTTCAAAT	Homolog of *T. aestivum PG-PLC1* (Cluster 586-contig 2 in FGAS) last 600 bp in ORF for RT-PCR, reverse
PG-PLC_L3	CCTCCTCCTGGCCCTGATGA	Homolog of *T. aestivum PG-PLC1* (cluster 586-contig 3) first 350 bp in ORF for RT-PCR, forward

Name	Sequence(5'-3')	description
PG-PLC_R3	GGCCGGGGTCGGAGTCC	Homolog of *T. aestivum PG-PLC1* (cluster 586-contig 3) first 350 bp in ORF for RT-PCR, reverse
PG-PLC_L4	ATGTTCCCACGCCTGTCAAA	Homolog of *T. aestivum PG-PLC1* (cluster 586-contig 5) first 650 bp in ORF for RT-PCR, forward
PG-PLC_R4	GCTCATATTGTGAGTCTTCCTTC	Homolog of *T. aestivum PG-PLC1* (cluster 586-contig 5) first 650 bp in ORF for RT-PCR, reverse
PI-PLC_L1	CGGACTCGGTGATGAAGAAG	*T. aestivum PI-PLC1* last 300 bp in ORF region for RT-PCR, forward
PI-PLC_R1	CATGAGGAGCTTGACGTTGG	*T. aestivum PI-PLC1* last 300 bp in ORF region for RT-PCR, reverse
PI-PLC_L2	TGCTAAAGAGGAGGATGCCT	Homolog of *T. aestivum PI-PLC1* (Cluster 3141-contig 1 in FGAS) first 600 bp in ORF region for RT-PCR, forward
PI-PLC_R2	GCCACCATCTGAGCACCG	Homolog of *T. aestivum PI-PLC1* (Cluster 3141-contig 1 in FGAS) first 600 bp in ORF region for RT-PCR, reverse
PI-PLC_L3	GGGTGCGGGCGGACTGC	Homolog of *T. aestivum PI-PLC1* (cluster 20268-contig 1 in FGAS) last 320 bp in ORF region for RT-PCR, forward
PI-PLC_R3	CATGAGGAGCCTGACGGACTT	Homolog of *T. aestivum PI-PLC1* (cluster 20268-contig 1 in FGAS) last 320 bp in ORF region for RT-PCR, reverse
Actin_F	GCTGATGGTGAAGACATTCA	*A. thaliana actin* gene for RT-PCR internal control , forward
Actin_R	CATAGCAGGGGCATTGAAAG	*A. thaliana actin* gene for RT-PCR internal control , reverse
P_4A	CTATATATTGCGATGAGGATAAAGTTAAGC	*At1g18070* last 500 bp in ORF region for RT-PCR, forward

Name	Sequence(5'-3')	description
P_4B	CAGGGACAGGGTTTACAATGC	*At1g18070* last 500 bp in ORF region for RT-PCR, reverse
P_6A	GATGTCTACAAGATTGGTGGTATTGG	*A. thaliana* EF-1α last 640 bp in ORF region for RT-PCR, forward
P_6B	AACCCTGTGGGAGCAAAGG	*A. thaliana* EF-1α last 640 bp in ORF region for RT-PCR, reverse
SAIL_LB	TTCATAACCAATCTCGATACAC	SAIL mutant lines T-DNA left border primer
142_LP	GAAAGAGAGAGCTCGAGAACCCG	*At1g18070* specific primer to verify SAIL_142 H_01 T-DNA insertion, forward
142_RP	GGGATTTATTTACAATGGTTGCCA	*At1g18070* specific primer to verify SAIL_142 H_01 T-DNA insertion, reverse
SALK_LBb1	GCGTGGACCGCTTGCTGCAACT	SALK mutant lines T-DNA left border primer
105834_LP	TTCCATCGTGTTTAACTTGATCC	*At1g18070* specific primer to verify SALK_105834 T-DNA insertion, forward
105834_RP	CCCTTGCTGTATCACTTGCAG	*At1g18070* specific primer to verify SALK_105834 T-DNA insertion, reverse
105836_LP	CCTTTACCTCAATGTCCTCGG	*At1g18070* specific primer to verify SALK_105836 T-DNA insertion, forward
105836_RP	TCTCAGATTTCCCTCAACTTGG	*At1g18070* specific primer to verify SALK_105836 T-DNA insertion, reverse

2. *J822* GENOMIC DNA CLONING

2.1. Wheat genomic DNA extraction

Genomic DNA in wheat was extracted from shoot tissue using a modified CTAB buffer protocol. Five gram of shoots from winter wheat cultivar Norstar was ground to powder with liquid nitrogen and was transferred to 50 ml falcon tube with 5 ml of 2X CTAB buffer (0.1M Tris-HCl, pH = 9.0, 1.4M NaCl, 20mM EDTA, pH = 8.0, 2g CTAB / 100ml (Sigma), and 0.5 % volume ß-mercaptoethanol) pre-heated to 65°C. The tube was vortexed for 30 seconds at low speed and then incubated at 65°C for 30 minutes. The sample was vortexed a second time and 10 ml chloroform/isoamyl alcohol (24:1) was added to the tube which was vortexed an additional 1 minute. The sample was centrifuged at 9,460 × *g* (relative centrifugal force) for 10 minutes, the aqueous phase (about 10 ml) was transferred to a new falcon tube; 1 ml NaOAc (3M, pH=5.2) and 6 ml Isopropanol (100%) were added and the sample was kept at -20°C overnight. Samples were then centrifuged at 9,460 × *g* for 20 minutes, and DNA pellet was formed at the bottom of the tube. The supernatants were discarded and the pellets were washed by 70% ethanol twice. The sample was recentrifuged at 9,460 × *g* for 1 minute, the ethanol was removed and the pellet was dried at room temperature for about 15 minutes. The genomic DNA was resuspended in 500ml nanopure H_2O.

2.2. *J822* genomic DNA cloning

Three pairs of primers (P_1A/1B, P_2A/2B and P_3A/3B) were used in an effort to isolate genomic clones of *J822*. A region corresponding to a portion of *J822* was successfully amplified by the primer pair P_3A and P_3B. The amplified fragments were subcloned to TOPO vector, and plasmid was purified from three positive colonies by miniprep. Three plasmids (D6, D8 and D12) were sequenced at the Génome Québec Innovation Centre at McGill University.

3. PLANT MATERIALS AND GROWTH CONDITIONS

3.1. Soil preparation

Equal volumes of perlite, vermiculite, peat moss and soil were mixed, then autoclaved at 121 °C for 45 minutes to eliminate the risk of insects and fungus. Water was added to the autoclaved soil before wheat or Arabidopsis were planted.

3.2. Wheat plant cold acclimation

The spring wheat *Triticum aestivum* L. cv Quantum and the winter wheat *T. aestivum* L. cv Clair were used in the study of 1 day to 36 days cold acclimation. All 12 plant samples were obtained from Dr. F. Sarhan's Laboratory in Université du Québec à Montréal (UQAM).

The winter wheat *T. aestivum* L. cv Norstar, LT_{50} - 19°C, was used in the study of early time course cold acclimation. The seeds were germinated in the greenhouse (26 ± 1°C) for 7 days. At the end of this period, aerial part of the control seedlings were

harvested, and meanwhile, plants for cold acclimation were transferred to the growth chamber (Conviron E15) at 4°C. The chamber maintained a wide spectrum fluorescent light (875 micromoles/m^2/s) with 16 hours of light and 8 hours of darkness. Plants were harvested every 2 hours up to 24 hours of cold acclimation. The harvested material was frozen immediately in liquid nitrogen, packed in aluminum foil and stored at -80°C.

3.3. Comparison of young and old tissue in wheat

The winter wheat *T. aestivum* L. cv Norstar was used in the study. The seeds were germinated and grown in the greenhouse (26 ± 1°C) for 23 days when the youngest leaves were larger than 5 cm. The oldest and youngest leaves were harvested and processed separately. The tissues were stored at -80°C for RNA extraction.

3.4. Salt, drought and NO treatment of wheat

The winter wheat *T. aestivum* L. cv Norstar seeds were germinated in a growth chamber for 10 days. The temperature was maintained at 25 ± 1 °C during the day (14 hours photoperiod) and 22 ± 1 °C during the night. The salt treatment was performed by watering the plants once a day for 3 days with a solution of: 150 mM NaCl, 15 mM $CaCl_2$. Control plants were treated with water. Aerial parts of seedlings for control and treatment were harvested at the same time and stored at -80 °C before RNA extraction.

The drought treatment was performed by withholding water for 4 days. Control plants were watered daily. Control and treatment plants were harvested at the same time, then were divided into two samples each. Samples for RNA extraction were stored at -80 °C. The second sample was used to estimate water content on the tissue. Plant material

was used to measure fresh weight, then dried at 70 °C for 48 hours and reweighed. A comparison of the fresh and dried weight in the control and drought treated plants was used to calculate the relative water content.

For the nitric oxide treatment, the plants were transferred to a new chamber which was maintained under the same conditions of light and temperature as the chamber used for germination and early growth. Controls were maintained in the original growth chamber. Plants were sprayed with 100 uM sodium nitroprusside (SNP, a nitric oxide donor) 5 times, once every 2 hours. The tissues of control and treatment were harvested at the same time and stored at -80 °C for RNA extraction..

3.5. Arabidopsis plant cold acclimation

Arabidopsis thaliana Colombia ecotype Col-0 obtained from the Arabidopsis Biological Resource Center (ABRC) was used in this study. The seeds were cold treated (stratified) for 4 days at 4 °C after imbibition to synchronize germination, and then sown on soil in 3 inch pots. The plants were grown in the laboratory under a wide spectrum fluorescent light with 16 hours of light and 8 hours of darkness at an average temperature of 22°C for 21 days. At the end of this period, the aerial part of seedling for control plants were harvested, the plants for cold acclimation were transferred to growth chamber at 4 °C with 16 hours light and 8 hours darkness. These plants were cold treated for 1, 3 and 6 days, respectively. The aerial part of cold treated and untreated seedlings were harvested and stored at -80 °C for RNA extraction.

3.6. Screening Arabidopsis for T-DNA insertion mutations

T2 and T3 seeds for T-DNA insertional mutant lines were obtained from the Ohio State Arabidopsis Biological Resource Center (ABRC). The *A. thaliana* Colombia ecotype mutant lines SAIL_142 H_01, SALK_ 105834 and SALK_105836 are reported to have T-DNA insertions in or near the gene *At1g18070*. (*At1g18070* is the Arabidopsis gene with the highest degree of sequence similarity to *J822*). The planting procedure was the same as that described for controls of the cold acclimation experiment (Section 3.5.). When the seedlings had 6 or 7 rosette leaves, one leaf was cut for genomic DNA extraction. DNA extraction was the same as that described above for wheat (Section 2.1.). The genomic primers for verifying presence of T-DNA insertions were designed by SIGnAL (Salk Institute Genomic Analysis Laboratory) software (http://signal.salk.edu/tdnaprimers.2.html). PCR was carried out with genomic DNA using gene specific primers and left border primer of T-DNA to screen for plants which carried the predicted insertion. Positive plants were maintained to produce seed in order to obtain homozygous mutant lines. For the mutant line from which only the heterozygous plants were identified among the SAIL_142 H_01 T2 plants, T3 seed was harvested and planted again, homozygous T3 plants were identified and maintained to produce homozygous T4 seeds.

3.7. Characterization of Arabidopsis mutant lines

A. thaliana Colombia ecotype Col-0 obtained from ABRC and homozygous SAIL_142 H_01 mutant line were used in this study. The planting procedure was the same as that described above (Section 3.5.). Plants were divided into two groups. The

first group was used to observe the difference of phenotype. The second group was used to compare the difference of *At1g18070* gene transcript level in wild type and mutant plants. The harvesting of tissue was the same as described above for cold acclimation (section 3.5.).

All the plant cultivars and mutants used in this study are listed in the Table 2.

Table 2. List of plant materials and source

Species and cultivar or ecotype	Source/description
wheat *T. aestivum* L. cv Quantum	Dr. Fathey Sarhan, UQAM
wheat *T. aestivum* L. cv Clair	Dr. Fathey Sarhan, UQAM
wheat *T. aestivum* L. cv Norstar	Dr. Fathey Sarhan, UQAM
A. thaliana Colombia ecotype Col-0	Arabidopsis Biological Resource Center (ABRC)
A. thaliana Colombia ecotype SAIL_142 H_01 mutant line	T2 seeds obtained from ABRC
A. thaliana Colombia ecotype SAIL_142 H_01 mutant line	T3 seeds from heterozygous T2 plant in this study
A. thaliana Colombia ecotype SAIL_142 H_01 mutant line	T4 seeds(homozygous) in this study
A. thaliana Colombia ecotype SALK_ 105834 mutant line	T3 seeds obtained from ABRC
A. thaliana Colombia ecotype SALK_105836 mutant line	T3 seeds obtained from ABRC
A. thaliana Colombia ecotype SALK_105836 mutant line	T4 seeds(homozygous) in this study

4. RNA EXTRACTION

Total RNA from shoot tissues of wheat and Arabidopsis was extracted by using a modified TRIzol protocol. Approximately 2 g of each frozen tissue was placed in a plastic weighing boat with liquid nitrogen. The tissues were cut into small fragments and ground in a mortar until the liquid nitrogen has almost completely evaporated. Ten ml (5 ml/g of tissue) of TRIzol Reagent (Invitrogen) was added to each mortar and the tissue powder was homogenized completely. The samples were transferred to a 50ml falcon tubes. They were vortexed vigorously for 1 minute and incubated for 5 minutes at room temperature. One ml chloroform was added and tubes were capped securely. The tubes were shaken vigorously by hand for 15 seconds and incubated for 2 minutes at room temperature. The samples were transferred to 50 ml autoclaved polypropylene centrifuge tubes and centrifuged at 21,300 × *g* for 10 minutes. The upper aqueous phase was withdrawn and placed in a round-bottom polypropylene culture tubes and 0.5 volume of isopropyl alcohol was added to the tube which was capped securely. The tubes were inverted a few times and incubated at room temperature for 20 minutes. The samples were centrifuged at 9,460 × *g* for 10 minutes, the supernatants were discarded, 5 ml of 75% ethanol was added to each tube and the samples were incubated at room temperature for 15 minutes. The total RNA was collected by centrifuge at 9,460 × *g* for 5 minute, the ethanol was removed and the pellets were dried at room temperature for about 15 minutes. The pellets were dissolved into 500 ul formamide. The RNA samples were treated for 3 cycles of freeze-thaw at -20 °C and room temperature. At the last cycle, the samples were vortexed to complete dissolution. The samples were transferred to 1.5 ml microcentrifuge tube and stored at -20 °C.

5. RT-PCR

5.1. RNA precipitation

Fifty ul aliquots of RNA samples dissolved in formamide were transferred to 1.5 ml autoclaved microcentrifuge tubes. If the concentration of the sample was less than 200 ng/ul, the sample of the RNA was increased to 100 ul or more. One tenth volume NaOAc (3M, pH=5.2) was added to the tube and mixed well, then 2.5 volume 99% ethanol was added and mixed. The sample was stored at -20 °C for at least 20 min. The RNA sample was centrifuged at 12,800 × *g* and 4 °C for 15 minutes. The supernatant was discarded and pellets were washed with 750 ul 75% ethanol. The sample was centrifuged for additional 5 minutes at 12,800 × *g* and 4 °C, then the ethanol was removed and the sample was dried at room temperature for about 10 minutes. The RNA was dissolved in 26 ul molecular biology grade distilled water (DNAse, RNAse free, Invitrogen).

5.2. DNAse I treatment of RNA samples

The 26 ul RNA sample was transferred to 200 ul autoclaved PCR tube with 3 ul 10X DNAse I buffer and 1 ul DNAse I (2 unit/1 ul, DNA- free™ , Ambion) added. The sample was incubated at 37 °C for 30 minutes. The reaction was stopped by addition of 0.1 volume DNAse Inactivation Reagent (DNA- free™) and incubated at room temperature for 2 minutes with occasional mixing. The sample was centrifuged at 8,940 × *g* and 4 °C for 1.5 minutes. The supernatant was carefully removed and placed in a new tube for the further application.

5.3. RNA quality and quantification

RNA samples of 5 ul was mixed very well with 995 ul water (dilution factor = 200) in a 1.5 ml microcentrifuge tube. The concentration of nucleic acid was analyzed by UV-visible spectrophotometer (Cary 50Bio, VARIAN)

5.4. Reverse transcript (RT) reaction and cDNA normalization

The RT reaction was performed by Superscript™ First-strand Synthesis System for RT-PCR (Invitrogen). RNA samples of 5 ug was mixed with 1 ul oligo $(dT)_{12\text{-}18}$ (0.5 ug/ul) and 2.5 ul 10 mM dNTPs. Additional DEPC-treated water was added to the mixture in order to reach the volume of 25 ul. The sample was incubated at 65 °C for 5 minutes, and then placed on ice for at least 1 minute. Twenty-five ul of reaction mixture (5 ul 10X RT buffer, 10 ul 25 mM $MgCl_2$, 0.1 M DTT, 1 ul RNAse inhibitor and 4 ul DEPC-treated water) was added to the previous RNA/primer tube and was incubated at room temperature for 2 minutes. One ul of enzyme SuperScript™ II RT (50 units/ul) was added to the tube, mixed and incubated at room temperature for 10 minutes. The reaction of reverse transcript was performed at 42 °C for 50 minutes and terminated at 70 °C for 15 minutes. The cDNA sample was collected after brief centrifugation. In the last step, 1 ul of RNAse H (2 units/ul) was added and incubated at 37 °C for 20 minutes.

Experimental samples were PCR amplified by gene specific primers and 1 ul of each first strand (fs) cDNA sample as template. Primers for the ubiquitin gene and the conserved region of the alpha-tubulin gene family were used as internal controls for wheat, and primers for the actin gene were used as a control for Arabidopsis (primer

sequences in Table 1). The concentration of each cDNA sample was normalized by comparison of the intensity of each PCR product by gel electrophoresis and ethidium bromide staining for the products from control primer sets. The normalized cDNA samples were used for RT-PCR for comparison of gene transcript level. Bands are framed by hand and quantified using software Gene Tools (Version 3.00.22) and GeneSnap (version 4.00) provided by the manufacture.

The lack of DNA contamination of the RNA sample was verified by PCR amplification without reverse transcriptase treatment.

6. CHARACTERIZATION OF THE INTERACTORS OF J822

A phosphatidylglycerol specific phospholipase C (PG-PLC1), and a phosphoinositide-specific phospholipase C (PI-PLC1) were identified as proteins that interact with the *J822* gene product based on yeast two-hybrid results in the laboratory of Jean-Francois Laliberte (Institue Aramnd Frapier). The expression of these and related genes were evaluated by RT-PCR. The gene specific primers of *PG-PLC1* (PG-PLC_L1/R1) and *PI-PLC1* (PI-PLC_L1/R1) were designed based on the *T. aestivum* tentative contig sequences, TC265102, TC254393 from TIGR wheat gene index (Release 10.0) (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=wheat). The PCR primers for homologs of *PG-PLC1* (PG-PLC_L2/R2, L3/R3 and L4/R4), and the homologs of *PI-PLC1* (PI-PLC_L2/R2 and L3/R3) were designed from similar contig sequences from FGAS wheat EST databases. The contig sequences were aligned and

PCR primers were designed for regions that were gene specific among similar gene family members and for which high quality sequence was available.

7. DATA ANALYSIS

Basic Local Alignment Search Tool (BLAST, Altschul et al., 1990) services provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) were widely used in this study. This suite includes five programs which can compare nucleotide sequences (BLASTN), the amino acid sequences (BLASTP), a given nucleotide sequence translated in six reading frames to amino acid sequences with the sequence entries in protein databases (BLASTX), a given amino acid sequence with the nucleotide sequences in the DNA databases translated in six reading frames to amino acid sequences (TBLASTN), and the comparison of a given nucleotide sequence translated in six reading frames to amino acid sequences with the sequence entries in a nucleic acid databases translated in six reading frames to amino acid sequences (TBLASTX). These programs are also available to compare two individual sequences, which may be nucleotide or amino acid sequences and in order to calculate the identity and similarity (bl2seq) between the two sequences.

The Institute Genomic Research (TIGR, http://www.tigr.org/tdb/tgi/) wheat gene index, The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), Functional Genomics of Abiotic Stress (FGAS, https://bioinfo.uwindsor.ca/cgi-bin/abiotic/project.cgi) wheat ESTs databases are used to identify and analyze the genes used in this study and their homologs and to help design gene specific primers. European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/) ClustalW was used to multiple

sequences alignment and analysis of sequence structure. The software Primer3 (Rozen and Skaletsky, 2000) and Oligos© (1999-2001) were used to design and test all the primers in this study. Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress) "T-DNA Express" Arabidopsis Gene Mapping Tool was used to search *J822*-homolog *At1g18070* mutant lines and design gene specific primers for genomic DNA.

PART III. RESULTS

1. SEQUENCE ANALYSIS

1.1. Isolation of a *T. aestivum* full-length cDNA clone *J822*

Four cDNA clones (A7, A12, B3, and C9) that included the entire open reading frame (ORF) for *J822* were isolated by PCR with gene specific primers from wheat cDNA libraries. A7, A12 and B3 have identical nucleotide sequences. They have inserts of 1800 bp including 60 nucleotides upstream of the ATG initiation codon, a 1602 nucleotide ORF, which encodes a 533 amino acid protein, and 3'UTR sequence of 138 nt. The predicted protein has a molecular weight of 58.9 kDa and isoelectric point (p*I*) of 5.49. The complete nucleotide sequence of *J822* is shown in Figure 3. The full length clone C9 has 2 base pairs differences (nt 106 and 1352) from A7, A12 and B3 in ORF region. Two other clones (B11 and C12) had 60 and 68 bp deletions, respectively, in the 5' end of the ORF. These deletions do not appear to be the result of miss-priming during the PCR amplification, because the primer does not match beginning of the clone.

```
cagcccaaaaaccctagacgccgccgcgcccagctccctcgctcccctcggcccgccggg        60
Atggagcacgacgcgccgcccgccccctcgcaccacgcccaggacgacggcgccgtggac       120
 M  E  H  D  A  P  P  A  P  S  H  H  A  Q  D  D  G  A  V  D    20
Gactgggcgcgcgacgacgcggagccgtccgatcggcacgccgcccccgcggaggagagc       180
 D  W  A  R  D  D  A  E  P  S  D  R  H  A  A  P  A  E  E  S    40
Cccgagcccgcggacgccgccgccgcccccgcgccaccagcggaaggtgtcaatgacatt       240
 P  E  P  A  D  A  A  A  A  P  A  P  P  A  E  G  V  N  D  I    60
Cagtcatcacttcagtcgttggagttgaagacaaatgctcctgcgcatgaggatgttcaa       300
 Q  S  S  L  Q  S  L  E  L  K  T  N  A  P  A  H  E  D  V  Q    80
Atggtagcagatgaggaagaggaagaaaagcgccatataaatttggttttcatcggccat       360
 M  V  A  D  E  E  E  E  E  K  R  H  I  N  L  V  F  I  G  H   100
Gttgatgcggggaaatcgactgctggagggcaaatattgttcttgagtggtcaggttgat       420
 V  D  A  G  K  S  T  A  G  G  Q  I  L  F  L  S  G  Q  V  D   120
Gaccggaccatccagaaatatgaaaaagaagcaaaggataagagccgagaaagttggtat       480
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 K  S  Y  V  P  N  M  I  S  G  A  S  Q  A  D  I  G  V  L  V   200
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 I  S  A  R  K  G  E  F  E  T  G  Y  E  R  G  G  Q  T  R  E   220
Catgtactgcttgcaaaaactctaggtgttgctaagttggtagttgtcatcaacaagatg       780
 H  V  L  L  A  K  T  L  G  V  A  K  L  V  V  V  I  N  K  M   240
Gatgaacctacagtacaatggtcaaaagaaaggtatgatgaaattgaagggaagatgatt       840
 D  E  P  T  V  Q  W  S  K  E  R  Y  D  E  I  E  G  K  M  I   260
Ccttttctcagatcttcagggtacaatgttaagaaagatgtccagttcttgcctatttct       900
 P  F  L  R  S  S  G  Y  N  V  K  K  D  V  Q  F  L  P  I  S   280
Ggtctttgtggagccaatatgaagaccagaatggataaaagcatttgtagttggtggaac       960
 G  L  C  G  A  N  M  K  T  R  M  D  K  S  I  C  S  W  W  N   300
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Ccagtaaggctgccaattattgataaatataaagatatgggcacagtcgtaatgggaaaa      1080
 P  V  R  L  P  I  I  D  K  Y  K  D  M  G  T  V  V  M  G  K   340
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 V  K  V  T  G  I  N  L  D  E  K  K  V  R  R  A  G  P  N  E   380
Aatgtacgtgtcaaagtgtctggaattgaagaggaggatatcatggcaggttttgtactt      1260
 N  V  R  V  K  V  S  G  I  E  E  E  D  I  M  A  G  F  V  L   400
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 S  S  V  A  N  P  I  G  A  F  T  E  F  N  A  Q  L  Q  I  L   420
Gagttgcttgataatgctattttcactgctggttacaaggcagtgttacacatccactct      1380
 E  L  L  D  N  A  I  F  T  A  G  Y  K  A  V  L  H  I  H  S   440
Gttgtcgaggagtgtgagattgttgatctcatagaggaaattgacatgaagaaagcgaaa      1440
 V  V  E  E  C  E  I  V  D  L  I  E  E  I  D  M  K  K  A  K   460
Gtaactgacccaaagaaaaagaagaccaagaggaagcctctttttgtgaagaatggtgca      1500
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Gttgtagtttgccgcgtccaggtgactaatttgatatgcatagagaagttctctgatttc      1560
 V  V  V  C  R  V  Q  V  T  N  L  I  C  I  E  K  F  S  D  F   500
Cctcagcttggaaggtttactctacgaactgaaggcaagacaatagctgtaggcaaggtt      1620
 P  Q  L  G  R  F  T  L  R  T  E  G  K  T  I  A  V  G  K  V   520
Gttgatgttcctccagttggcaggtcaacgttttcagcttaagccaattttgacaggatg      1680
 V  D  V  P  P  V  G  R  S  T  F  S  A  -                     533
Atgggagaatgaagtggcgctgtctcgaaataggttttggaagcagtaagatcgattttt      1740
Ccagccaggctccatgagtcacttagcattagtttattgctgaggtccctttattgagtt      1800
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Figure 3. Nucleotide Sequence of *J822* and deduced amino acid sequence. The top line is nucleic acid sequence of *J822*. Bottom line represents deduced amino acid sequence. Numbers represent nucleotide and amino acid position.

1.2. Sequence comparison between J822 and related genes

Sequence comparison of J822 by BlastX to GenBank sequences in National Center For Biotechnology Information (NCBI) databases, showed the most similar gene was an unnamed protein from rice, the second, third and fourth most similar proteins were annotated as *EF-1 α* related GTP-binding protein, from *Nicotiana* and Arabidopsis. However, J822 had relatively low similarity (56%) to a known wheat translation elongation factor gene, *EF-1 α. J822* homologues in rice and Arabidopsis have 88% and 81% amino acid sequence similarity to wheat J822. They have only 57% and 58% sequence similarity to the known EF-1 α protein sequences in rice and Arabidopsis, respectively (Table 1.). EF-1 α is highly conserved between each species. EF-1 α from wheat has 98% and 97% sequence similarity to its homologues in rice and Arabidopsis. Furthermore, EF-1 α from rabbit has 85% similarity to wheat EF-1α. *J822* apparently does not have an ortholog in a non-plant species. The similarity and identity between wheat cDNA clone J822 and related genes are showed in Table 3.

1.3. Biophysical characteristics of wheat cDNA J822 protein

The molecular weight (Mw) and p*I* as predicted by the Compute pI/Mw Tool (http://ca.expasy.org/tools/pi_tool.html) showed large differences between J822 and EF-1 α. The Mw of proteins predicted from the sequences of J822 and its homologues are between 58.9 and 59.2 KDa, and p*I*'s are from 5.19 to 5.49, but EF-1 α Mw are approximately 50 kDa with p*I* higher than 9.

Table 3. Amino acid sequence identity and similarity between J822 and related genes

	J822	o-J822[a]	At-J822	W-EF-1 α	o- EF-1 α	At- EF-1 α	r- EF-1 α
J822	100	84[b]	71	37	37	37	37
o-J822	88	100	80	37	37	37	37
At-J822	81	87	100	37	37	37	37
w-EF-1 α	56	55	57	100	96	95	77
o- EF-1 α	58	57	58	98	100	95	77
At- EF-1 α	57	56	58	97	97	100	75
r- EF-1 α	54	55	56	85	85	84	100

a. o-J822, J822 homologue gene in rice; o- EF-1 α, rice elongation factor-1 α; At-J822, J822 homologue gene in Arabidopsis; At-EF-1 α, Arabidopsis elongation factor-1 α; w-EF-1 α, wheat elongation factor-1 α; r- EF-1 α, rabbit elongation factor-1 α;

b. numbers from column 2 to 8 represent amino acid sequence identity and similarity between pairs of gene products, values above the centre diagonal represent amino acid sequence identity (%) and numbers below the centre diagonal represent % similarity.

1.4. Sequence analysis of J822 protein

The amino acid sequence of wheat cDNA clone J822 was analyzed in order to infer its structure and function. EF-1 α contains three structural domains, an N-terminal EF-Tu GTP-binding domain, a C-terminal beta barrel domain, which is involved in binding to charged tRNA, and a C-terminal beta barrel domain involved in binding to both charged tRNA and binding to EF-Ts. Studies of the crystal structure of rabbit EF-1 α have identified a critical **His 296** as well as other amino acids as part of the tRNA binding site, which are involved in aminoacyl-tRNA binding. The other conserved amino acids include **Met 294, His 295, His 349, Cys 363, His 367, Met 404** and **Met 410** (Kinzy et al., 1992). Analysis of the tomato EF-1 α, the first EF-1 α identified in vascular plants, showed that the functional domains of EF-1 α are conserved both in sequence and their relative position between mammals and plants (Pokalsky et al., 1989).

GTP-binding proteins are mainly classified into three groups, which include alpha subunits of heterotrimeric GTP-binding proteins, the small monomeric GTP-binding proteins with a molecular mass of 20 to 30 kDa and the alpha subunit of polypeptide chain elongation factors (EF-1-α), which catalyzes the binding of aminoacyl t-RNAs to the A site of the ribosome. The GTP-binding domain from these three groups share a consensus sequence composed of three elements **GXXXXGK, DXXG** and **NKXD** (Dever et al., 1987). The first and third elements were known to form a P-loop and to be involved in GTP interaction (Pokalsky et al., 1989). Figure 4 shows amino acid sequence comparisons between J822 and EF-1 α from rice, Arabidopsis, tomato, wheat and rabbit. The three consensus elements in the GTP-binding domain are highly conserved in J822, which indicates that *J822* probably encodes a GTP-binding protein. However, the His

296 and other important amino acids involved in aminoacyl tRNA binding in rabbit EF-1 α are conserved in the plants EF-1 α's, but not in J822. It can be inferred that J822 does not have tRNA binding functional domains in this region, although alignments showed some similarity between J822 and EF-1α.

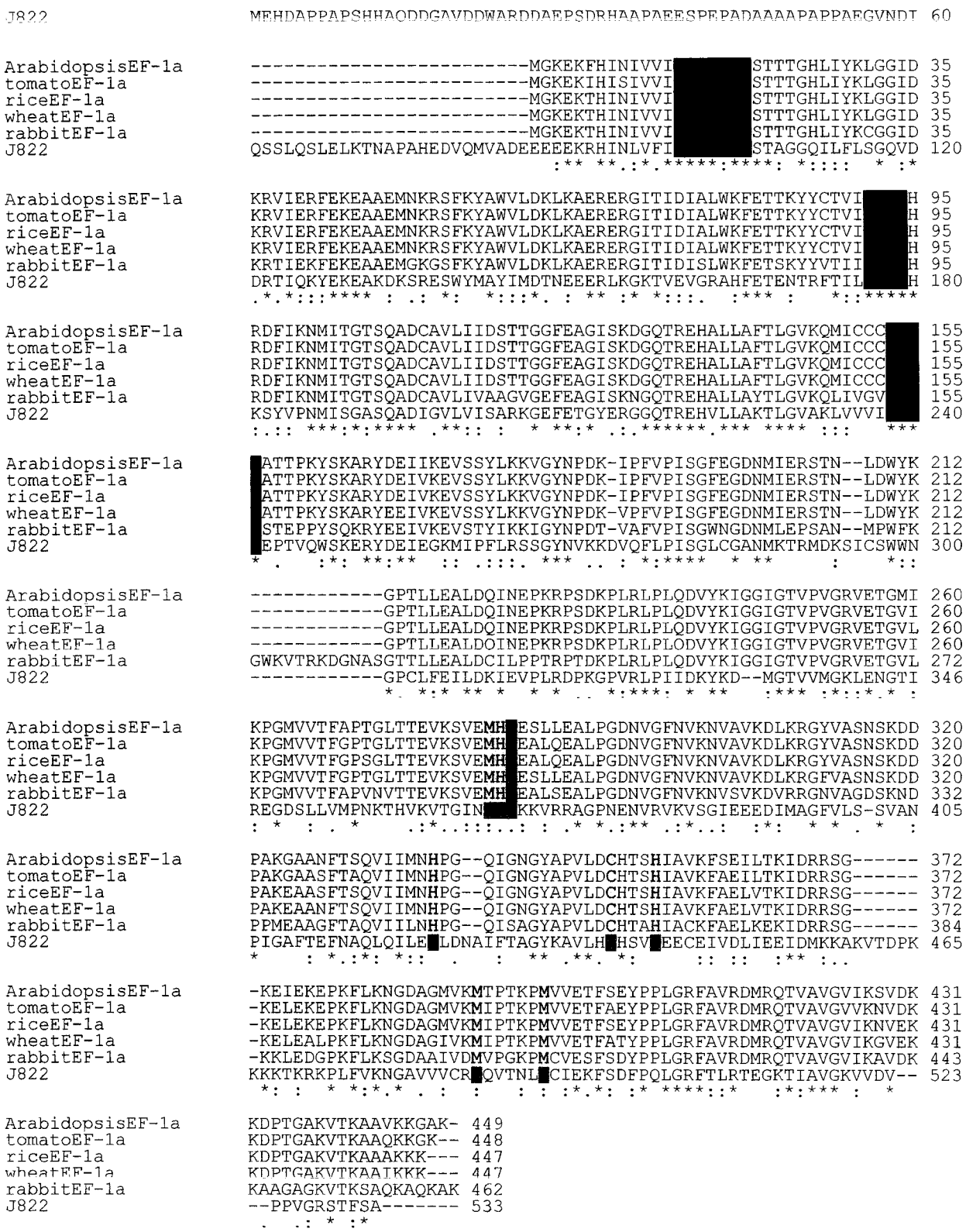

Figure 4. Multiple sequences alignment of J822 and EF-1 α from Arabidopsis, tomato, rice, wheat and rabbit. CLUSTAL W (1.82) was used for multiple alignments of the sequences. Letters highlighted in pink indicate three consensus elements in the GTP binding domain. The H highlighted in red is His 296 in rabbit EF-1 α, which is a tRNA binding site. Letters highlighted in blue indicate conserved amino acids that are involved in tRNA binding. Letters highlighted in green indicate tRNA binding site and reagents not conserved in J822.

2. CHARACTERIZATION OF *J822*

2.1. Wheat cDNA clone *J822* is induced by cold

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to analyze the effects of cold acclimation on the transcript levels of *J822* and to compare changes in gene expression in winter wheat and spring wheat cultivars. *J822* was more strongly induced in the highly freezing tolerant winter wheat cultivar Clair during cold acclimation than in the more sensitive spring cultivar Quantum. Differences in band intensity indicated that *J822* was induced 2.7 fold at day 1 and over 10 fold from day 3 to 36 in winter wheat. It was induced about 2.5 fold in spring wheat, much less than that observed in winter wheat (Figure 5).

The measurement of gene induction during the first 24 h of cold acclimation indicated *J822* was generally induced from 4 to 24 hours. However, there are no significant differences between control and samples taken at the 6 and 10 hours time points. *J822* was strongly induced from 12 hours until 24 hours (Figure 6).

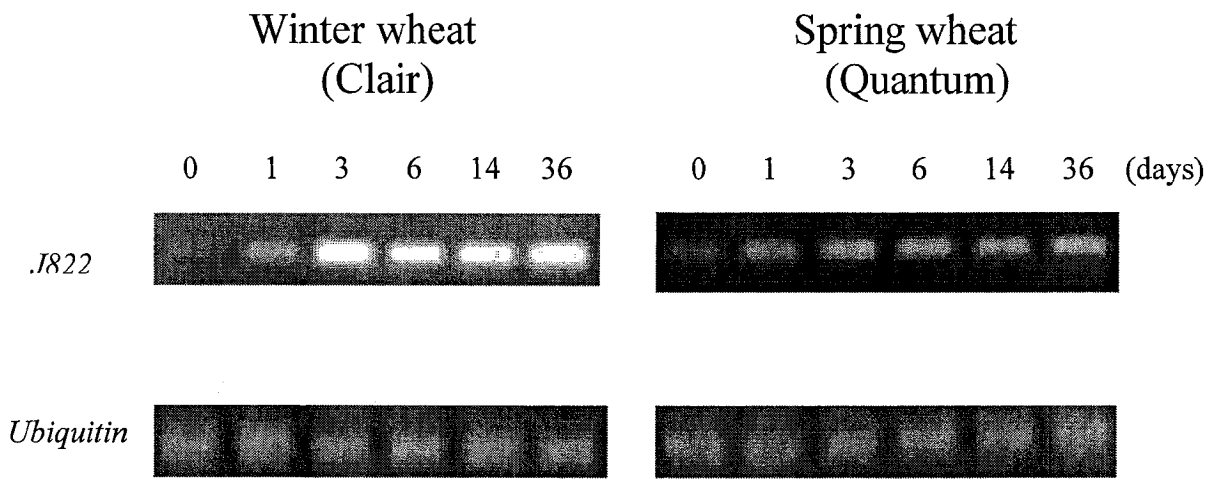

Figure 5. The expression of *J822* in winter and spring wheat cultivars during cold acclimation. *J822* transcript levels are determined by RT-PCR analysis with total RNA samples from shoots of winter wheat cultivar, Clair, and spring wheat cultivar, Quantum. The plants were treated at 4°C. *Ubiquitin* was used as a internal control to normalize the total RNA level in each sample.

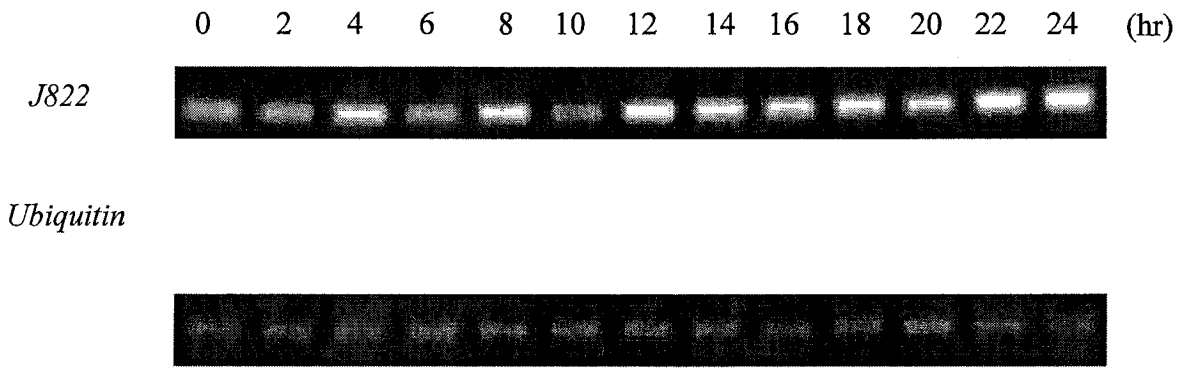

Figure 6. The expression of *J822* during the first 24 hours of cold acclimation. RT-PCR was used to determine *J822* transcript level, using total RNA samples from shoots of winter wheat cultivar Norstar. The plants were treated at 4°C. *Ubiquitin* was used as a control gene to normalize total RNA levels.

2.2. *J822* is induced by drought, salt and nitric oxide treatments

Different environmental stress responses in plants share common signaling pathways. There are many reports that protein kinases and phospholipases are regulated by both cold and salt stress (Seki et al., 2002; Krebs et al., 2002; Shinozaki et al., 2003). Although little is known about function of nitric oxide (NO) in plants, it has been demonstrated that endogenous NO is induced by drought and salt stress and that exogenous application of NO affect plant growth (Lamattina et al, 2003). *J822* was strongly induced by salt and drought stresses, and was slightly induced by NO treatment. *J822* was induced 3 fold in plants subjected to dehydration to 50% relative water content, and were induced 2 fold under high salinity conditions. There was an increase in *J822* levels in plants treated with SNP, a compound which releases NO (Figure 7), since it is less than 2 fold change, it is difficult to quantify accurately by RT-PCR.

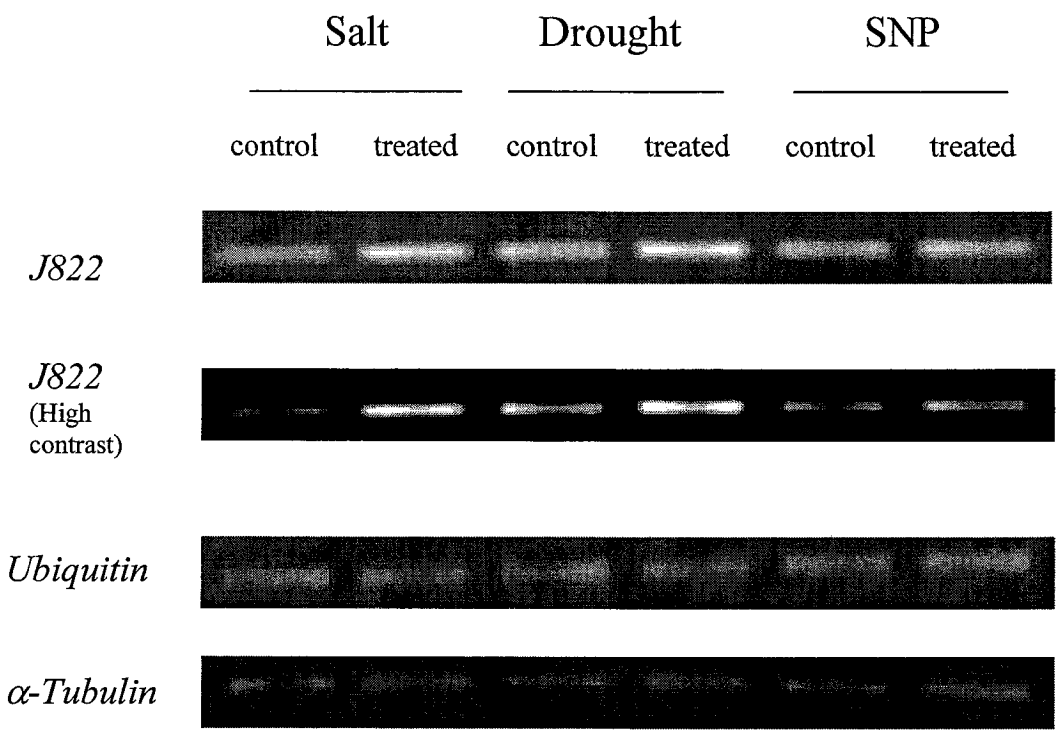

Figure 7. The expression of *J822* in response to salt, drought and extrogenous nitric oxide treatment. RT-PCR was used to compare *J822* mRNA level using RNA samples from the winter wheat cultivar Norstar. Plants were untreated (Control) or treated by salt (150 mM NaCl + 15 mM $CaCl_2$) for three days, drought treated to 50% relative water content, and by sodium nitroprusside (SNP, a NO donor) 100 uM treatment for 1 day with 5 sprayings. *Ubiquitin* and *α-tubulin* are used as controls to normalize the mRNA levels in the samples.

2.3. Comparison of the expression of wheat cDNA clone *J822* and *EF-1 α*

J822 has some sequence similarity to EF-1 α, but sequence analyse provided evidence that the function of J822 may be difference from EF-1 α. It has been demonstrated that *J822* was strongly induced by cold treatment. RT-PCR analysis indicated that the transcript level of *EF-1 α* does not change during cold acclimation up to 6 days (Figure 8). The *EF-1 α* mRNA is relatively abundant in plant tissues and higher levels of mRNA were found in developing tissues such as young leaves and green fruit compared to the mRNA levels observed in older tissues. The increased levels of *EF-1 α* mRNA, therefore, appear to correlate with higher levels of protein synthesis in developing tissues (Pokalsky et al., 1989). This level of expression of *J822* was observed to be the same in young and old tissues. In contrast, the expression of *EF-1 α* is higher in younger leaves than in older leaves as was previously reported (Figure 9).

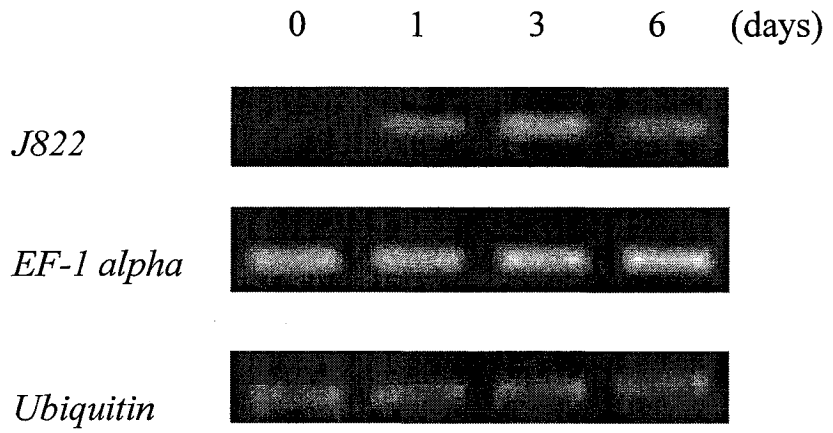

Figure 8. The expression of *J822* and *EF-1 α* during cold acclimation. RT-PCR analysis was used to determine mRNA level of *J822* and EF-1 α genes. Plants were cold acclimated up to 6 days, and total RNA from the winter wheat cultivar Clair was analyzed. *Ubiquitin* was used as a control to normalize total mRNA level.

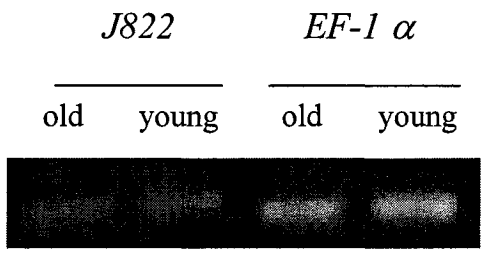

Figure 9. The expression of *J822* and *EF-1 α* in old and young leaves. RT-PCR analysis was used to determine mRNA level of *J822* and *EF-1 α* genes. RNA was extracted from same plants but different leaves. The old leaf sample was from first emerged leaf (first leaf counting from the base of the plant). The young tissue was taken from the fourth leaf (last leaf to emerge).

2.4. J822 interacts with Phospholipase Cs

A yeast-two-hybrid screen was done in collaboration with Dr. Laliberté's Laboratory (Institut Armand-Frappier, Laval) in order to identify proteins that interact with J822. Two proteins were shown to interact with J822 in this assay: a phosphatidylglycerol specific phospholipase C (PG-PLC1) and a phosphoinositide-specific phospholipase C (PI-PLC1). Several wheat EST sequences were available for *PG-PLC1* in GenBank and the full-length cDNA sequence was represented by the tentative contig TC265102 from The Insititue for Genetic Research (TIGR) Wheat Gene Index (Release 10.0) (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=wheat). In the FGAS wheat EST databases this gene is represented by Cluster 586 contig 4. There are 8 tentative contigs related to *PG-PLC1* with a match above an E-value -20 in TIGR rice databases.

PI-PLC1 is represented by multiple wheat ESTs in GenBank that cover the approximately 400 amino acids of coding sequence. The region covered by the TC is approximately 2/3 of the carboxyl end of the protein predicted from the rice gene with highest level of sequence similarity (GI:12698878) which has 598 amino acids. The wheat ESTs for this gene have been assembled into TC254393 in TIGR databases. There are 6 contigs related to *PI-PLC1* with a match above an E-value -20 in TIGR rice databases.

2.4.1. The wheat *PLCs* were induced by cold stress

Both *PG-PLC1* and *PI-PLC1* were strongly induced by cold treatment from 1 day to 14 days. At 36 days of cold acclimation, *PG-PLC1* remained strongly induced, but the mRNA levels for *PI-PLC1* began to decline (Figure 10).

Sequences were identified for three genes with high sequence similarity to *PG-PLC1* corresponding to FGAS cluster 586-contigs 2, 3, and 5. They have 91, 96, 97 % sequence identity, respectively with *PG-PLC1*, and were referred to as *PG-PLC2*, *PG-PLC3* and *PG-PLC4*. Sequences for the tentative contigs are presented in Appendix 1. *PG-PLC2* was induced by cold acclimation as observed for the *PG-PLC1*. *PG-PLC4* is weakly expressed, but induced, whereas no RT-PCR product was detected for *PG-PLC3* (Figure 11). *PG-PLC3* is represented by a single EST sequence in FGAS with only 341 nt of high quality sequence in the EST. Alignment of *PG-PLC3* and TC265102 showed there are only two regions that can be used to design gene specific primers. Verification by software Primer3 indicated high self complementarity and severe primers dimer occurrence. The lack of an RT-PCR product may well have been caused by poor primer design or very low gene expression in the plants.

PI-PLC1 is represented by FGAS tentative contig, cluster 3141-contig 2. *PI-PLC1* has 95 % sequence identity to cluster3141-contig 1, and 79% identity to cluster 20268-contig 1, which are referred to as *PI-PLC2* and *PI-PLC3* respectively (sequences *PI-PLC2* and *PI-PLC3* are presented in Appendix 1). RT-PCR results with gene specific primers for these genes showed, *PI-PLC2* is the same as that for the *PI-PLC1*, both of which are strongly induced by cold acclimation from 1 day to 14 days, but induction is reduced at 36 days of cold acclimation. *The PI-PLC3* gene is also strongly induced, and

expression of its mRNA level remained high for up to 36 days of cold treatment (Figure 12).

2.4.2. The *PLCs* are induced by salt, drought stress and NO treatment

There are many reports that PLCs were regulated by salt and drought stress in plants, but there are no reports on the regulation of phospholipases by exogenous nitric oxide. In this experiment, the *PG-PLC1* was induced by salt, drought and NO treatment. *PI-PLC1* was induced by drought and NO, but not by salt stress (Figure 13).

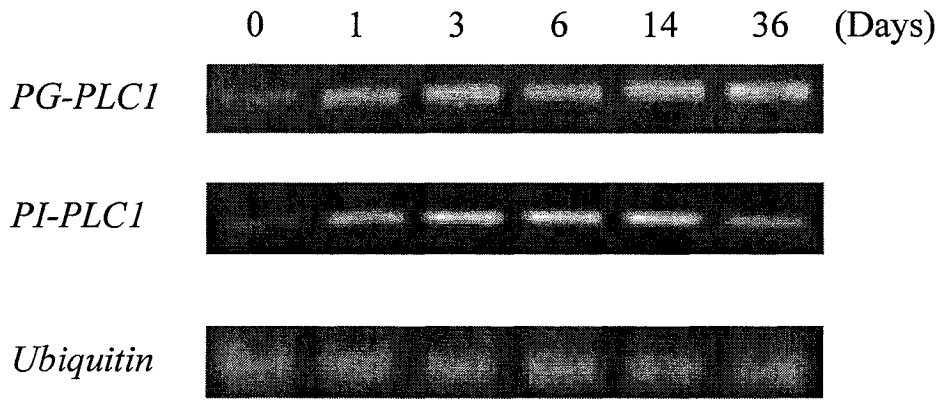

Figure 10. The expression of *PG-PLC1* and *PI-PLC1* during cold acclimation. The transcript levels for two *phospholipase C* genes were determined by RT-PCR. Total RNA samples were from shoots of the winter wheat cultivar Clair; plants were treated at 4°C. *Ubiquitin* was used as a internal control to normalize the total RNA level in each sample.

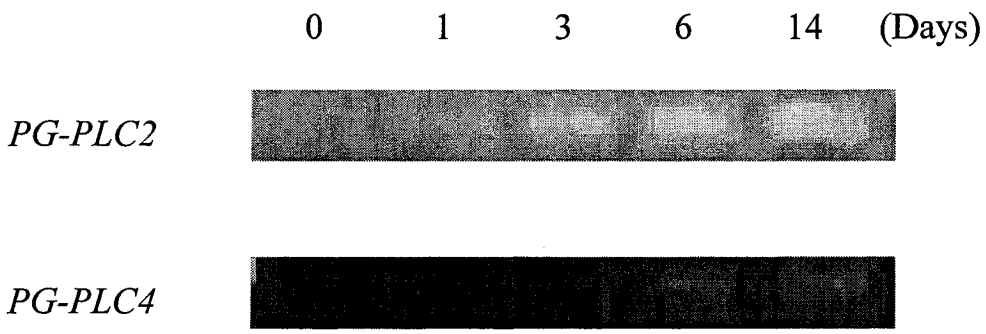

Figure 11. The expression of *PG-PLC1* related genes during cold acclimation. The transcript levels of *PG-PLC1* related genes were determined by RT-PCR. Total RNA samples were from shoots of the winter wheat cultivar Clair; plants were treated at 4°C.

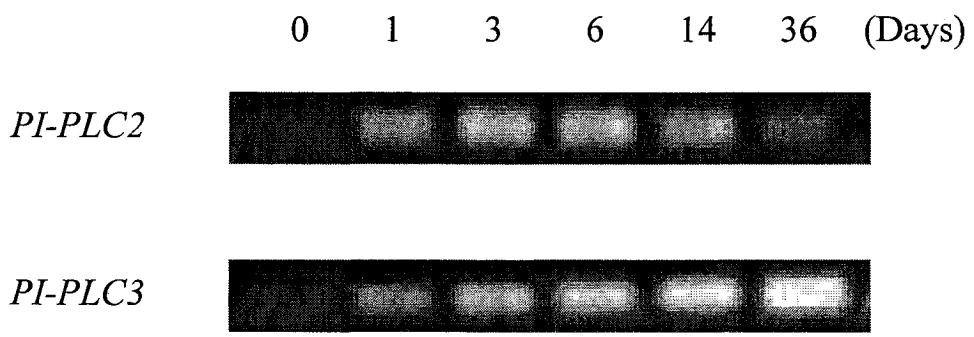

Figure 12. The expression of *PI-PLC1* gene family members during cold acclimation. The transcript levels of *PI-PLC2* and *PI-PLC3* genes were determined by RT-PCR. Total RNA samples were from shoots of the winter wheat cultivar Clair; plants treated at 4°C.

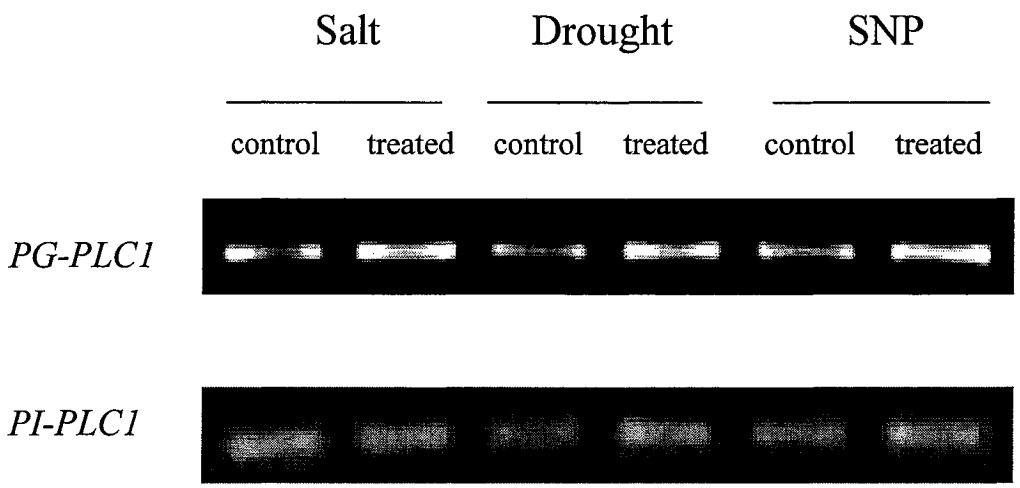

Figure 13. The expression of *PG-PLC1* and *PI-PLC1* in salt, drought stress and exogenous nitric oxide treatment. RT-PCR was used to determine mRNA levels for two PLCs. Samples of total RNA from the winter wheat cultivar Norstar were analyzed. Plants were either untreated or treated by salt (150 mM NaCl + 15 mM $CaCl_2$), drought (to 50% relative water content) and sodium nitroprusside (SNP) 100 uM treatment. SNP releases the signaling compound NO.

2.5. Wheat *J822* genomic DNA cloning

Attempts to obtain a full length genomic clone of *J822* were not successful, however a 3' end partial-length genomic DNA was amplified and cloned. Three positive clones (D6, D8 and D12), the longest of which was 630 nucleotide long, were sequenced. Alignment of the three clones sequence indicated that D6 has only one nt difference with D8. D12 has 24 nt substitutions and six gaps for a total 23 nucleotide differences relative to D8 (Figure 14).

The comparison of *J822* partial genomic DNA and cDNA sequences indicates that the genomic sequence has three exons and two introns. The only one unmatched nucleotide between D6 and D8 is located in the first exon, there are three unmatched nucleotide between D12 and D8 located in the three exons, other differences were distributed over two introns (Figure 14 and 15).

There are eight wheat EST sequences obtained from the GeneBank wheat EST databases, which contain the portion of *J822* that were sequenced in this study. Multiple alignment of these sequences indicated that D8 is identical to cDNA-A7. Both D6 and cDNA-C9 have 1 nt unmatched to D8 and A7 in this region, the differences are probably caused by PCR induced errors. D12 have 3 nucleotide mismatches to cDNA-A7 and D8. One maybe caused by a PCR error, the other two were found in two independent clones (D12 and gi_22303739) (Figure 15).

Alignment of 13 sequences gave evidence for four *J822* related genes in the wheat genome. The first gene was represented by 6 independent clones (D6, D8, cDNA-A7, cDNA-C9, gi_70961057 and gi_70961496). The second gene was represented by 3 independent clones (gi_9742470, gi_25145813 and gi_20309210). The third gene was

represented by 2 independent clones (gi_38153187 and gi_70962829). The fourth gene was represented by two independent clones (genomic-D12 and gi_22303739) (Figure 15). Shared polymorphisms in the independent clones for the first two candidates support the hypothesis that these are clones of different genes. Evidence also supports the existence of two additional gene copies, however there are fewer clones with high quality sequences to confirm the latter two candidates. *T. aestivum* is a hexaploid species, thus the vast majority of genes are expected to have at least three copies. The presence of clones for orthologs from the three ancestral genomes in a EST database is dependent on the size of the EST data base for the species, and the level of expression for the gene and thus its representation in the EST collection. With more than 550,000 sequences, the wheat EST database is one of the largest plant EST collections.

The Blastn comparison of the *J822* cDNA sequence to TIGR wheat databases showed that there are two closely related contigs. One, TC251157, is 100% identical to *J822*, another one, TC251155, which is 98% identical to *J822* (412 nt identical from total 420 nt). Such high sequence similarity indicates that the two TC represent orthologous genes from two of the ancestral genomes.

Sequence comparison of the partial genomic clone with the most similar genes in Arabidopsis and rice showed a similar presence of two introns which are conserved in the closest Arabidopsis homolog, *At1g18070*, but the closest rice homolog has only one of these introns (Figure 16).

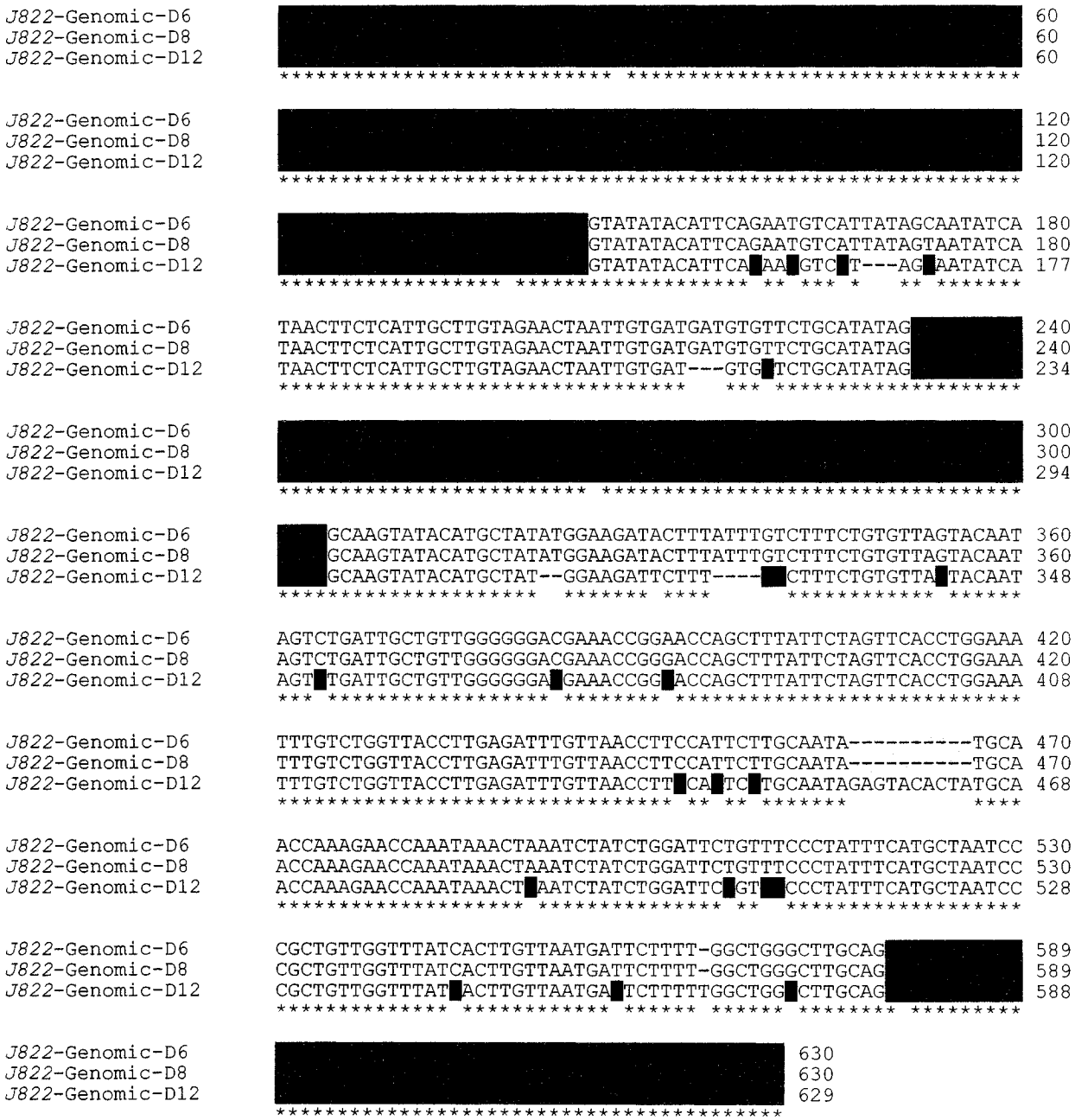

Figure 14. Sequences alignment of three partial *J822* genomic DNA clones. CLUSTAL W(1.82) was used to align D6, D8 and D12, three *J822*-like genomic DNA clones. The three exons are highlighted in green. There are two intervening introns. Nucleotide differences are highlighted in pink. Gaps in the alignment are highlighted in blue.

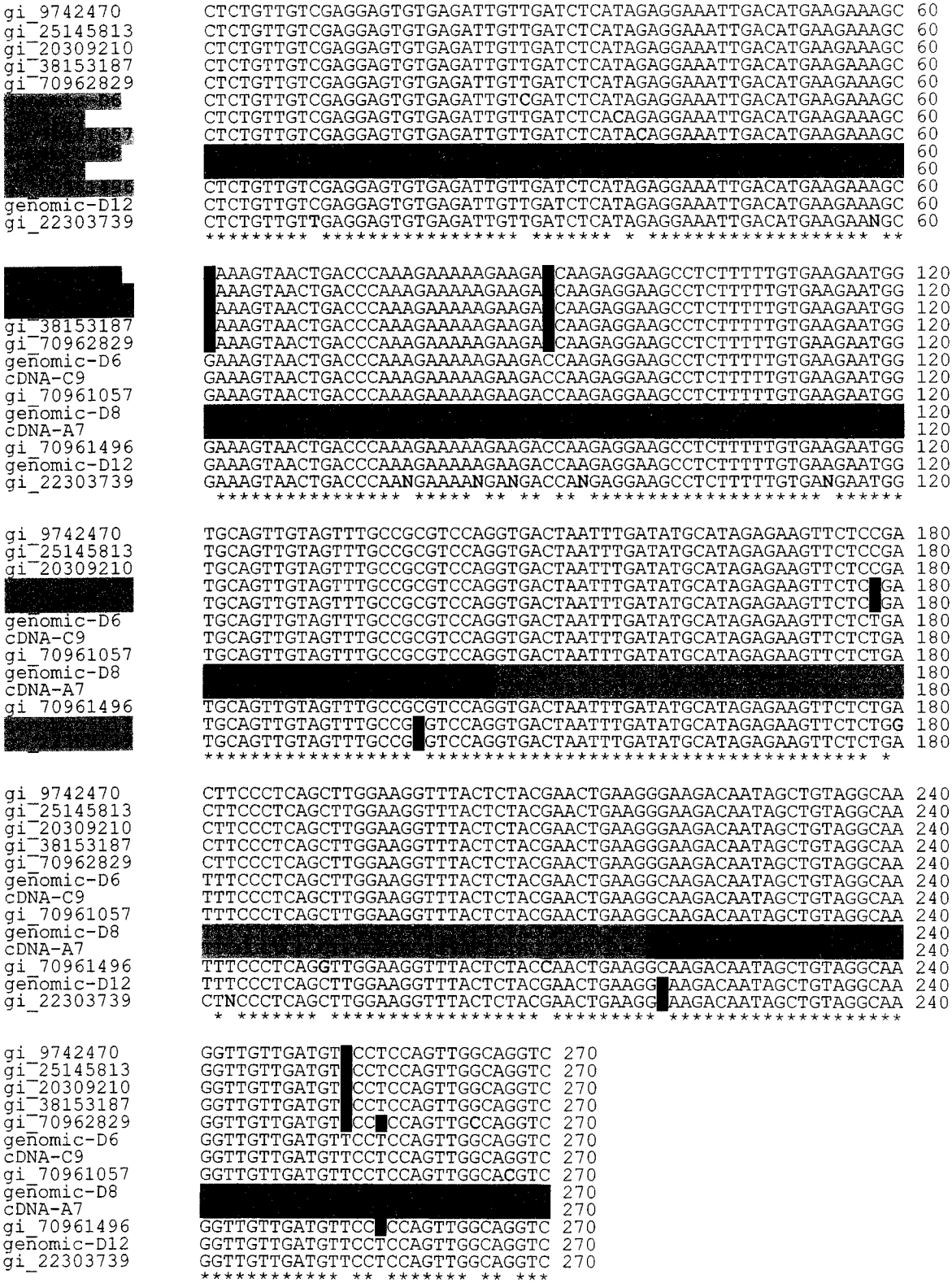

Figure 15. Multiple sequence alignment of *J822*-related sequences. CLUSTAL W (1.82) was used to align two *J822* cDNA (A7 and C9), three exons from genomic DNA clones, D6, D8 and D12 and 8 EST sequences from GeneBank databases. The letters highlighted in green-yellow-green indicate three exons. The letters highlighted in blue indicate sequencing or PCR error, in pink indicate sequence variation. The ID highlighted in the left side indicates at least 2 independent clones came from same gene.

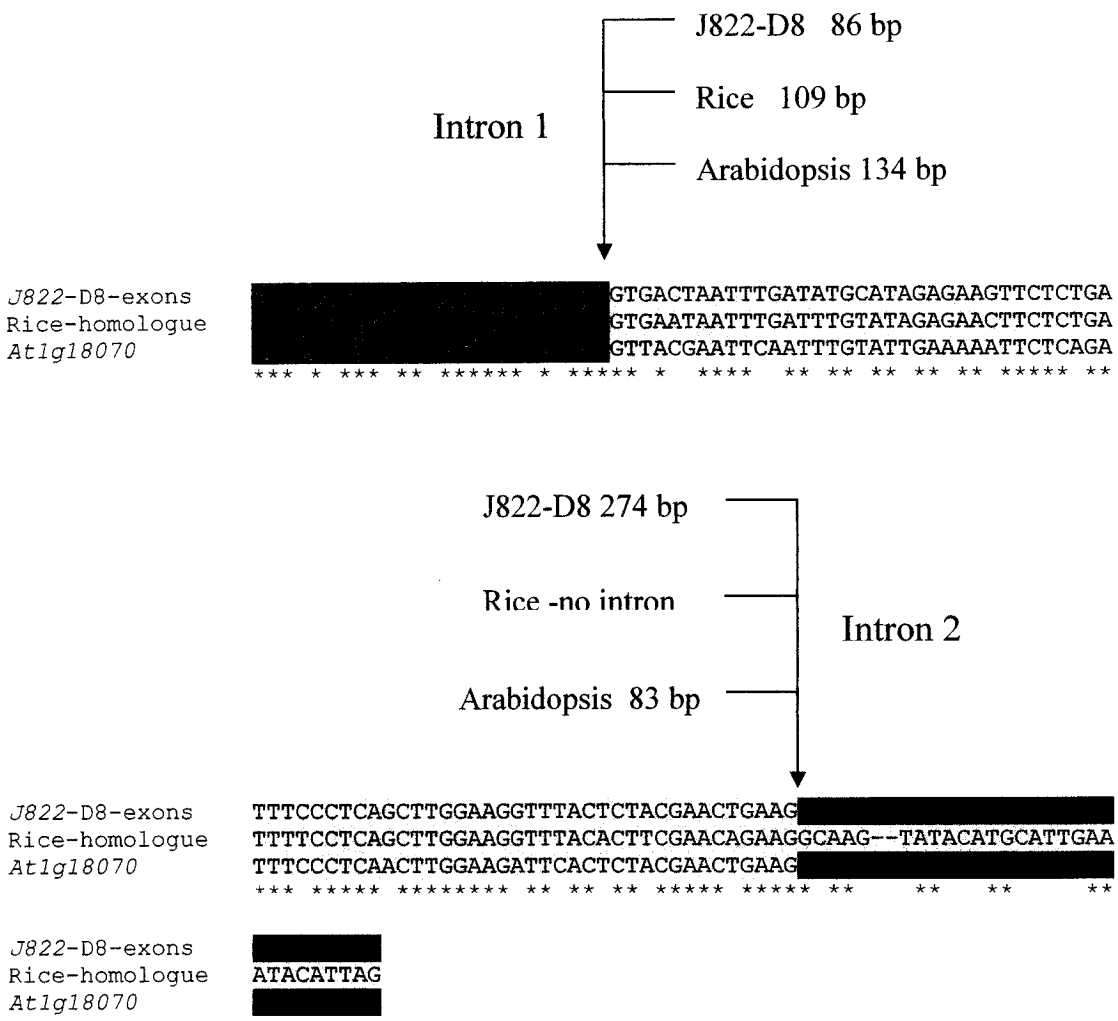

Figure 16. Comparison of intron/exon junctions for *J822* and homologs in rice and Arabidopsis. The partial sequence of *J822* genomic clone D8-exons was aligned with homologus gene sequences in rice and Arabidopsis. Black arrows indicate intron position and the length of the introns are indicated. The letters highlighted in green-blue-pink indicate *J822* and Arabidopsis have two introns in this region, and those highlighted in green-blue indicate that rice only has one intron.

3. CHARACTERIZATION OF A *J822* HOMOLOG IN ARABIDOPSIS

As a model plant, *A. thaliana* has several advantages for plant research. It is the first plant whose genome was sequenced in entirety, 28,000 genes have been identified in its genome and it is possible to study cold stress induced genes based on publicly available sequences. In this study, the sequence of J822 amino acid was used to search the Arabidopsis genomic databases. The gene which is most similar to *J822* in *A. thaliana*, *At1g18070*, has 71% amino acid sequence identity and 81% similarity. Characterization of this gene will be helpful to understand J822 function. *At1g18070* is a single copy gene in Arabidopsis genome. It contains 124 bp in 5' UTR region, a 1599 nucleotide ORF region, which encodes a predicted protein of 532 amino acids. There are 409 nucleotides in 3' UTR region. The deduced protein has a molecular weight of 59.2 KDa and a p*I* of 5.19. Gene expression analysis of *At1g18070* by RT-PCR using gene specific primers showed that it was strongly induced by 1 day to 6 days cold acclimation (Figure 17).

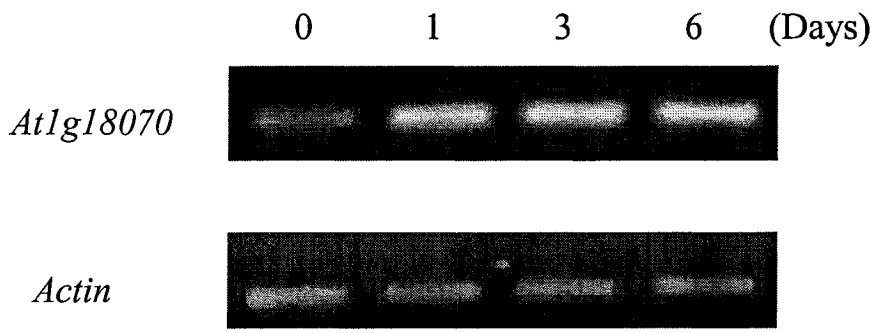

Figure 17. The expression of *At1g18070* gene during cold acclimation. RT-PCR analysis was used to measure mRNA levels of *At1g18070* during cold treatment up to 6 days. Total RNA from the Columbia-0 ecotype was analyzed. *Actin* was used as control gene to adjust total RNA levels of each sample.

3.1 T-DNA insertional mutant for *At1g18070*, SAIL_142_H01

One of the most direct ways to study a gene's function is to block its expression. T-DNA insertion is one of commonly used approaches to achieve this goal. In Arabidopsis, there are T-DNA insertion mutant collections available in three research centres (Arabidopsis Biological Resource Center, ABRC in the USA; Max Planck Institute for Plant Breeding Research, GABI-Kat in Germany; Institut National de la Recherche Agronomique, INRA in France), where mutations near a gene of interest can be screened by web based searches. When a mutant line is obtained, the precise location of the T-DNA insertion within a gene needs to be determined and homozygous mutant plant need to be experimentally verified before studying the function of this gene.

SAIL_142_H01 is a T-DNA mutant line generated in the Syngenta Arabidopsis Insertion Library (SAIL) collection. The T-DNA is 7,541 bp insertion and is located near the 5' UTR region of *At1g18070* gene based on information available in the SALK (Salk Institute genomic analysis Laboratory) database. PCR screening of 24 T2 plants with gene specific primers indicated that two plants were heterozygous for the insertion and the other 22 plants did not have insertions. This is unexpected since the T-DNA carries a BASTA-resistance gene. T1 transformed plants were identified by selection on BASTA. Three quarters of T2 plants would be expected to have at least one copy of the T-DNA insertion, if all seeds survived from the selection.

The size of the PCR amplification product generated in the heterozygous plants was used to determine the site of insertion. A PCR fragment between a gene specific and the left border primer specific for the T-DNA insertion was approximately 590 bp (Figure 18, A). There are 371 bp between the left border primer and the end of the insertion, and

the gene specific primer was located 154 bp downstream of the predicted start of transcription, so the insertion should be located 65 bp upstream of the start of transcription (Figure 18, B).

PCR screening of twenty T3 plants identified 4 plants homozygous (No.2, 8, 10 and 13), and 9 plants heterozygous for the insertion (No.3, 4, 6, 7, 11, 15, 18, 19 and 20) and 7 plants no insertion (No.1, 5, 9, 12, 14, 16 and 17) (Figure 19). Comparison between wild type and the T-DNA mutant did not detect any differences in phenotype and transcript level for *At1g18070* (Figure 20). An insertion 65 bp upstream of the start of transcription can be expected to affect gene expression. It has been reported that an insertion located upstream of the 5' UTR of EIN2 gene in Arabidopsis disrupts gene expression (Alonso et al., 1999), so the results for *At1g18070* are unexpected. More experiments should be done to verify these results.

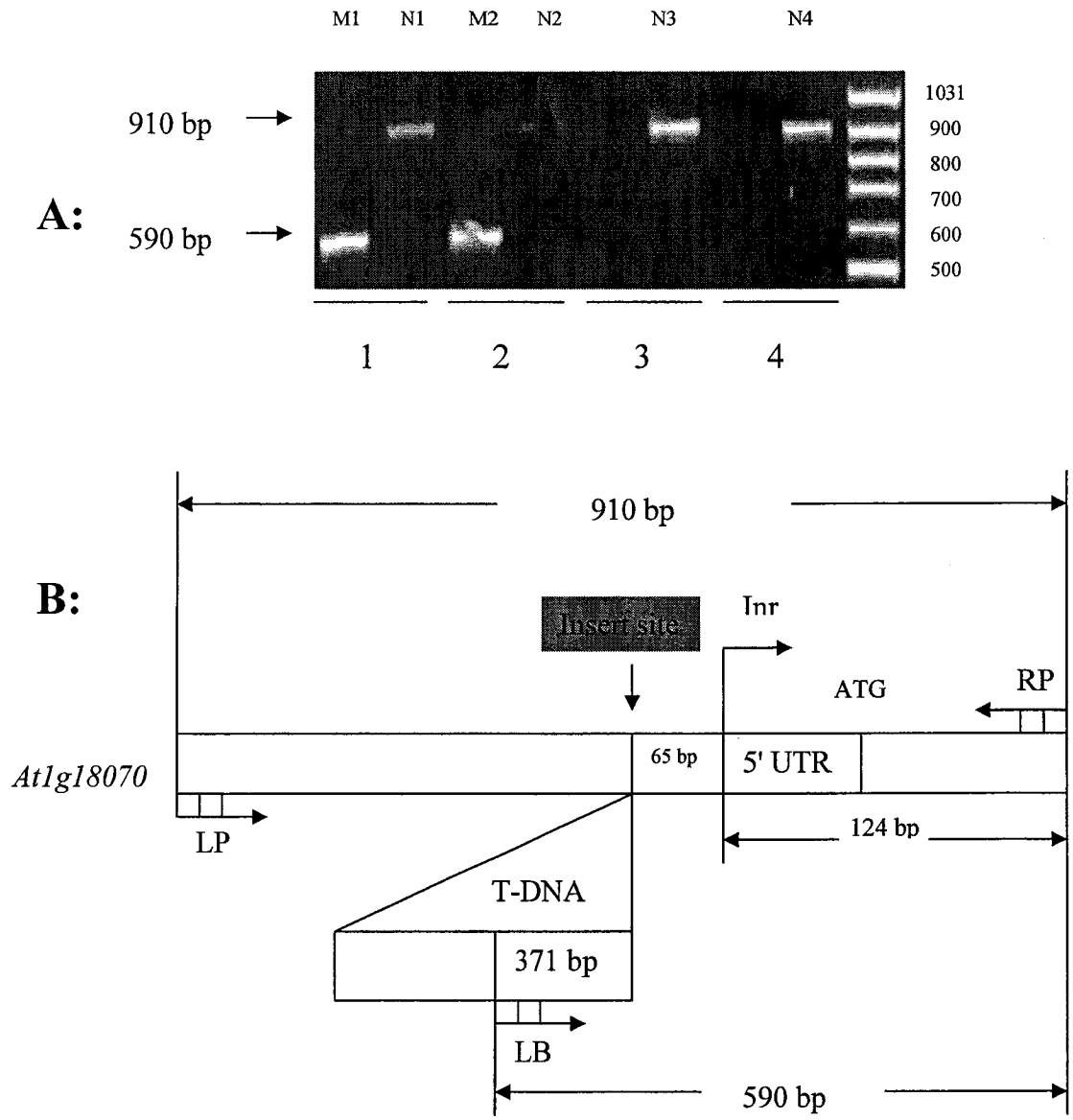

Figure 18. ***At1g18070*** **T-DNA mutant SAIL_142 H_01 line insertion located.**
(A) Homozygous mutant plants identified by PCR screening. The numbers under the gel represent different plants assayed. Plants 1 and 2 appear to be heterozygous, and 3 and 4 have no insertion. M1 and M2 indicate the products of left boarder primer of the T-DNA (LB) and right gene specific primer (RP), N1, N2, N3 and N4 indicate the products of the two gene specific primers (LP, RP). The right lane has DNA size markers. The numbers on the left side indicate the sizes of the products.
(B) The analysis of T-DNA insert site is shown on a model of the 5' end of *At1g18070* gene. LP- gene-specific left primer; LB- left border primer in T-DNA; RP- gene-specific right primer; ATG- start codon; 5'UTR- 5' end untranslated region; T-DNA- insertion fragment; the numbers under and inside the gene indicate the size of corresponding fragment.

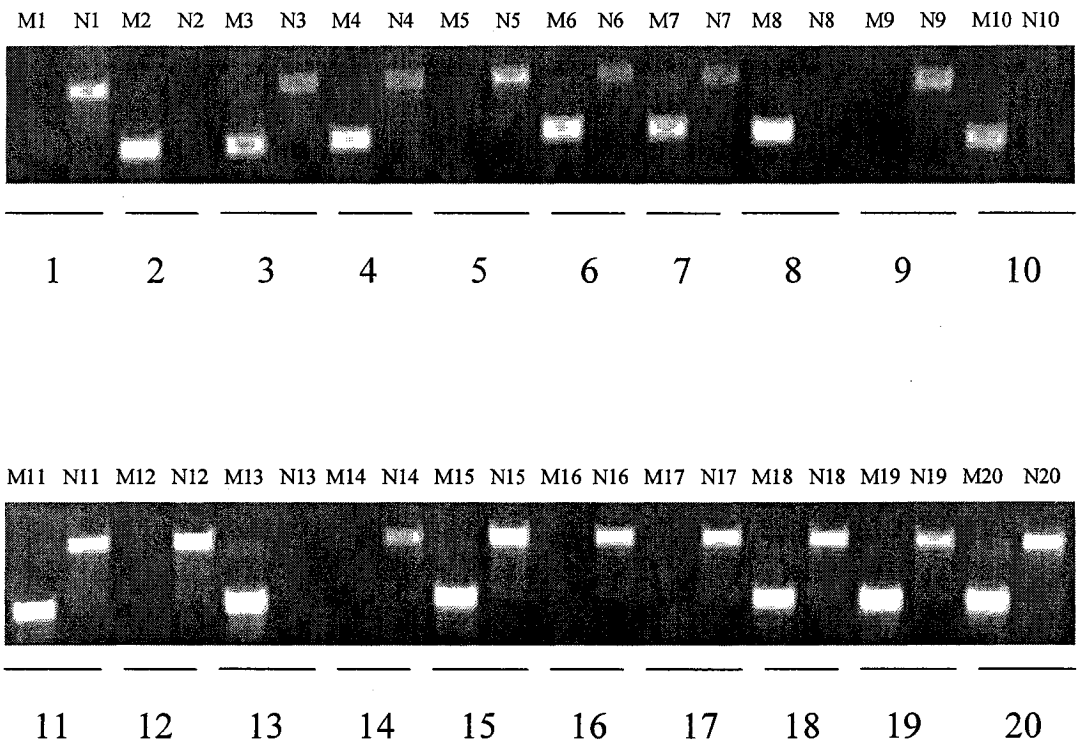

Figure 19. ***At1g18070*** **SAIL-142 H_01 T-DNA homozygous mutant isolation.** PCR amplified genomic DNA from different 20 plants T3 (N0.1-No. 20), derived from a single heterozygous plant T2 plant. M1-M20 indicate the products of left border primer and gene-specific right primer. N1-N20 indicate the products of gene-specific left primer and right primer. Plants with only M product are homozygous, those with only N product have no insertion. Plants with PCR products from both M and N primer pair are heterozygous of the insertion.

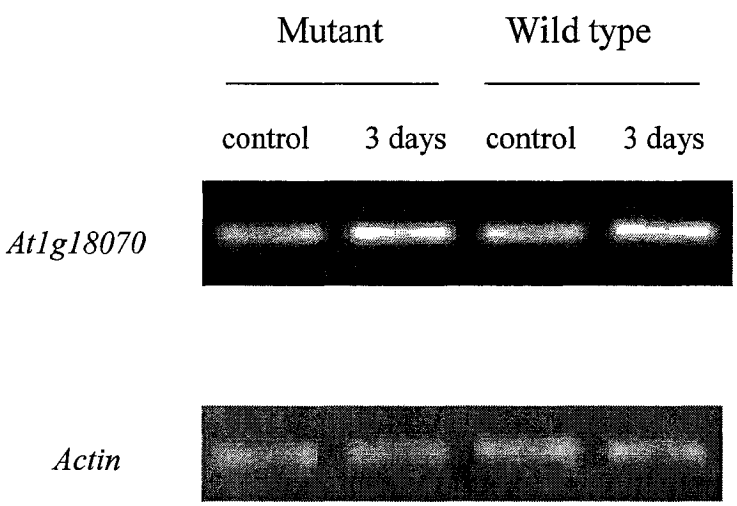

Figure 20. The expression of *At1g18070* in wild type and mutant plants during cold acclimation. RT-PCR was used to compare mRNA level in the mutant and wild type plants in response to cold treatment. Total RNA samples from wild type (Columbia-0) and SAIL_142 H_01 mutant line were analyzed. Plants were untreated (control) or treated at 4°C for 3 days. Actin was used as a control to normalize the mRNA levels in the samples.

3.2. SALK_105834 and SALK_105836 mutants

Both SALK_105834 and SALK_105836 T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (ABRC, Colombus, Ohio). T-DNAs were expected to be inserted near the 3'UTR based on information in the SALK database. PCR analysis of 40 T3 plants from the SALK_105834 line detected no plants with an insertion near *At1g18070*. There were 4 homozygous plants isolated from 20 T3 plants for the SALK_105836 mutant line. The homozygous plants have normal leaf number, leaf shape and growth rate. The size of the PCR amplification product generated in the homozygous plants was used to determine the site of insertion. A PCR fragment generated from a gene specific primer and the left border primer specific for the T-DNA insertion was approximately 650 bp (Figure 21, A). The gene specific primer was located 187 bp upstream stop codon and there are 110 bp between the left border primer and the end of the insertion. The insertion is therefore located 350 bp downstream of the gene stop codon, in the 3' UTR region of the gene (Figure 21, B).

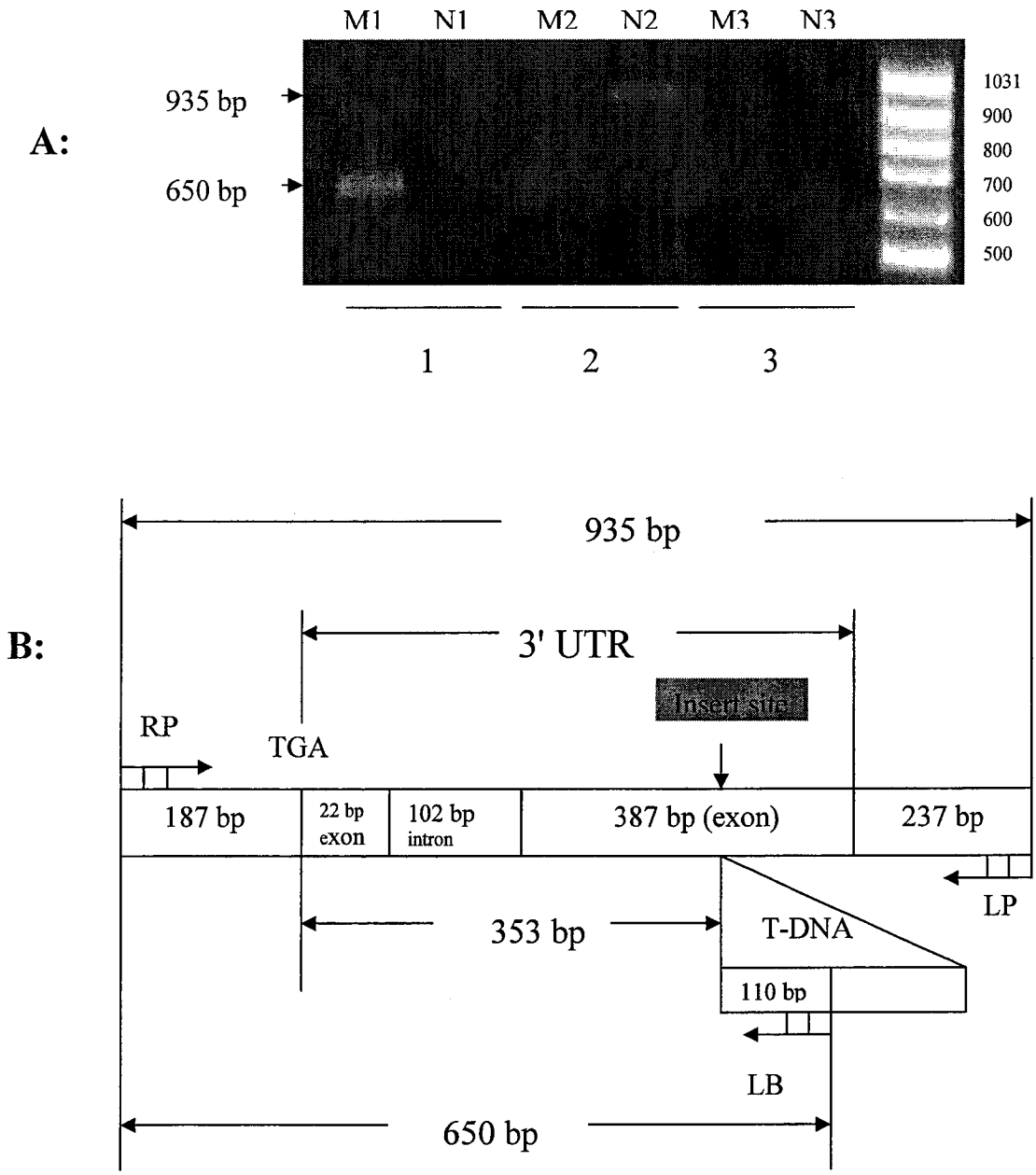

Figure 21. Localization *At1g18070* T-DNA mutant SALK_105836 line insertion. (A) M1, M2 and M3 indicate the products of left border primer for the T-DNA and a gene-specific right primer. N1, N2 and N3 are products from gene-specific left primer and right primers. Plants 1 and 3 are homozygous and plant 2 is heterozygous. The right lane has DNA markers. The numbers on the left side indicate the size of each product. (B) The analysis of T-DNA insert site is shown on a model of the 3' end of the *At1g18070* gene: LP- gene-specific left primer; LB- left border primer; RP- gene-specific right primer; TGA- stop codon; 3'UTR- 3' end untranslated region; T-DNA- insertion fragment; the number inside and under the gene indicate the size of corresponding fragment.

PART IV. DISCUSSION

In this study, full-length cDNA and partial genomic DNA clones for the cold induced wheat gene *J822* were obtained. J822 was classified as a putative GTP-binding protein due to highly conserved GTP-binding protein consensus elements. *J822* transcript level was shown to be more strongly induced during cold acclimation in the freezing tolerant winter wheat cultivar than in the more freezing sensitive spring wheat cultivar. This suggests a possible relation between the expression of *J822* and the capacity of each genotype to develop freezing tolerance. Yeast two hybrid screening indicated that the *J822* gene product interacts with a phosphatidylglycerol-specific phospholipase C and a phosphoinositide-specific phospholipase C. This is a novel contribution to the characterization of stress-induced signaling in wheat, an important crop species.

1. *J822* is a cold induced gene

The transcript level of *J822* was demonstrated by RT-PCR analysis to be strongly upregulated by cold treatment. It was cold induced after 2 hours and more strongly induced after 10 hours of cold treatment. The early response indicates that J822 may be active in the initial signaling events in response to cold. Low temperature stress induces the transcript level of two group of genes in wheat. The first group is transient in nature and reaches the highest levels of induction within the first 24 hours of low temperature exposure and then decreases to the basal levels. This group is hypothesized to include genes that are important in the initial signaling events controlling the response to low

temperature. The second group of genes are induced rapidly and maintain high expression levels for several weeks. Cold tolerant cultivars normally maintain persistently high levels of expression for the second class of response genes whereas sensitive cultivars do not (Danyluk et al., 1991). The very high transcript levels for *J822* that are maintained for up to 36 days of low temperature treatment suggests that J822 may be a regulator of the persistently upregulated cold responsive genes.

Several factors can affect mRNA levels, in addition to higher levels of transcription, decreased rates of mRNA degradation can lead to higher levels of mRNA. Regulation of G-proteins also function at the protein level in which activation and inactivation is controlled by GTP hydrolysis. Thus additional studies should explore the mechanisms of regulation of the *J822* gene product.

2. *J822* is induced by other environmental stresses

Many genes have been reported to be induced by multiple environmental stresses, including cold, salt, and drought. The existence of crosstalk in abiotic-stress-responsive signaling pathways has been discussed by several authors (Seki et al., 2002; Krebs et al., 2002; Shinozaki et al., 2003). In this study, the results showed, *J822* was strongly induced by salt and drought treatment, and slightly induced by NO treatment, suggesting that the regulation of *J822* is influenced by crosstalk between different stresses signaling pathways.

3. *J822* encodes a putative GTP-binding protein

GTP-binding proteins were initially classified into three groups including EF-1 α. Although J822 has 56% amino acid similarity to wheat EF-1 α, sequence analysis and comparison of gene expression in response to cold acclimation suggest it encodes a putative GTP-binding protein, and that it is not EF-1 α. J822 does not have significant similarity to other two groups of classical GTP binding proteins. Recently, several unique types of GTP-binding protein, which are not members of the aforementioned three groups and called unconventional GTP-binding protein, have been reported (Assmann, 2002). In this new group, three families in plants were identified, extra-large G proteins (XLG), root hair defective proteins (RHD) and developmentally regulated G proteins (DRGs). J822 has little similarity to these three families, so it may represent a new protein family.

GTP binding proteins are activated by GTP and inactivated when GTP is hydrolyzed to GDP. Presumably the novel GTP binding proteins are subject to the same regulation. This investigation of GTP regulation of J822 is a significant question to be addressed in future work.

Among genes that respond to cold, salt and drought stresses at the transcriptional level, some encode products that directly protect plant cells against the stresses, and others regulate gene expression and signal transduction in abiotic stress responses (Bray et al., 2000). There are many reports of GTP-binding proteins that are involved in signaling in plant cells (Millner, 2001), so it's possible that J822 plays a role in stress signal transduction. This hypothesis is based only on sequence analysis and gene

expression, so biochemical and molecular experiments are needed to confirm J822 GTP-binding function and its possible role in signaling.

4. J822 protein could interact with target proteins

The yeast two-hybrid system was used to identify proteins with which J822 might interact. J822 was found to associate with a PG-PLC and a PI-PLC in this assay. In mammal systems, phospholipid signaling is initiated by the binding of hormones to a receptor which triggers activation of alpha subunit of heterotrimeric GTP-binding protein. This activates PLC in the hydrolysis of PIP_2 to DAG and IP_3. In plants, to date, there have been no reports of the direct interaction of GTP-binding proteins and PLCs in the stress induced signaling pathway. However, there are reports that both GTP-binding proteins and PLCs are involved in the early steps of signal transduction in plants (Legendre et al., 1993; Pandey and Assmann, 2004). PLCs are well-established signal transduction proteins in plants. There are many reports suggesting that PLCs regulate cold or other stress induced signaling pathways, that involve IP_3 and PA, the products of PIP_2 hydrolysis by PLCs (Munnik and Meijer, 2001; Ruelland et al., 2002).

Our data suggest that J822 is involved in cold induced signal transduction by interaction and regulation of PLCs in wheat. Further studies on the relationship between J822 and the PLCs, and other proteins, is important for the characterization of stress-induced signal transduction pathways in wheat.

In this study, the results of RT-PCR analysis demonstrated that the two interactors of J822 were strongly induced by cold, drought, and NO stresses. This suggests that there is crosstalk in the signaling pathways of cold, drought and NO response in the

regulation of the PG-PLC and PI-PLC. In addition, the RT-PCR results showed the different cold induction patterns between the *PG-PLC* and the *PI-PLC*. Both the *PG-PLC* and *PI-PLC* are strongly induced from day 1 to day 14 of cold treatment. At 36 days of cold treatment, the *PG-PLC* is induced strongly and *PI-PLC* is only weakly induced. This suggests that there may be different regulatory mechanisms affecting the genes encoding these proteins.

5. *J822* homolog in Arabidopsis is induced by cold stress

The Arabidopsis genome was the first plant genome sequenced in its entirety. It is an important resource to study cold stress genes discovered in other species through the identification of orthologous genes. In this study, *At1g18070* was found to have high sequence similarity to J822. Characterization of the *J822* homologue in Arabidopsis will be helpful to further the understanding of the function of J822, and the search of the Arabidopsis genome databases indicated that it is a single copy gene. The scarcity of mutants among the T-DNA insertion stocks for this gene suggests that it has a critical function in the plant. RT-PCR analysis has demonstrated that *At1g18070* was strongly induced by cold stress. The gene induction of *At1g18070* suggests that it functions in Arabidopsis in a manner similar to that in wheat.

6. Whether J822 has an important role in freezing tolerance remains to be determined

Two T-DNA mutant lines obtained from ABRC and the identification of homozygous mutants in this project were used to study At1g18070 function. The mutant,

SAIL_142 H_01, has an insertion close to the start of the gene transcription that should disrupt the gene. But, RT-PCR results did not detect any change in the expression of *At1g18070* in this line. This is difficult to explain because the plants have been verified by PCR and the primers for RT-PCR are specific and not likely to amplify other RT products in the genome. These results should be verified by additional experiments. The site of the mutation should be sequenced to determine the accurate location of the insertion.

The mutant SALK_105836 has an insertion in the last exon in the 3' UTR of *At1g18070*. The 3'UTR is sometimes associated with mRNA stability, so this mutation should affect the expression of the gene. In the future, analysis of *J822* expression and observation of the phenotype in this line will be performed.

In this study, we have gained important evidence that *J822* is regulated by cold acclimation and other environmental stresses and that it is likely involved in signal transduction. In the future, more studies are needed to investigate the function of this gene in relation to environmental stress tolerance.

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Apendix 1

Tentative contig sequences for wheat phospholipase C's

1. *PG-PLC2* sequence (Cluster 586-contig 2, singleton in FGAS)

CTTCGACAGCCTCGAGGAGGATGGCCTCTCCTTCGGCATCTACTACCAGAACATCCCGGC
CACGCTCTTCTACCAGAGCCTCCGCCGCCTCAAGCACCTCGTCAAGTTCCACCAGTACAG
CCTCAAGTTCAAGCTCGACGCCTCGCGGGGCAAGCTGCCCAACTACGTCGTCATTGAGCA
GAGGTACTTCGATTGCAAGGAGTTCCCTGCCAACGACGACCACCCGTCGCACGACGTCGC
CAGGGGCCAGAGGTTTGTTAAGGAGGTCTATGAGACGCTGCGAGCCAGCCCGCAGTGGAA
CGAGACGGCTCTCATCATCACCTATGATGAGCATGGTGGCTTCTATGACCATGTCCCCAC
GCCCGTCGTCGGGGTGCCCCAGCCCGACGGGATTGTTGGTCCTGACCCTTACTACTTCAA
ATTCGACCGACTTGGGGTGCGCGTGCCCAGCTTCCTCATCTCCCCCTGGATTGAGAAGGG
CACTGCGATCCATGAACCAAATGGTCCATTTGAAAACTCACGATATGAGCATTCATCCAT
TCCTGCAACCGTAAAGAAGCTATTTAATTTACGTGCTAACTACCTGACAAAGAGGGATGC
ATGGGCCGGGACCTTTGAG

2. *PG-PLC3* sequence (Cluster 586-contig 3, singleton in FGAS)

ATGGCCGCCGCCGGAGAGCGGCGCCTCCTCGCGTGCCTCCTCCTCCTGGCCCTGATGATC
TCGGCGCACTGCCTGGACGGCGCGGGCCACCACGGCCCGCGCGTGAAGCGGCGCCGCAAG
AAGCGCGAGATCCACTCCCCCGTCAAGACGGTGGTGGTGGTGGTCATGGAGAACCGCAGC
TTCGACCACGTCCTCGGCTGGCTCCGCGCCGGCCGCCCCGACATCGACGGGCTCACCGGC
ACGGAGTCCAACCGCCTCAACGCGTCCGACCCGGCCTCCCCGGAGATCTTCGTCACCGAC
AAGGCCGGCTACGTGGACTCCGACCCCGGCCACGGCTTCGA

3. *PG-PLC4* sequence (Cluster 586-contig 5, assembly sequence in FGAS)

TACAGCCTCAGGTTCAAGCTCGACGCCGCCAGGGGCAAGCTCCCCAATTACGTCGTCATC
GAGCAGAGGTACTTTGACTGCAAGGAGTTCCCCGCCAACGACGACCACCCGTCGCACGAC
GTCGCCAGGGGCCAGAGGTTTGTCAAGGAGGTCTACGAGACGCTGCGGGCGAGCCCGCAG
TGGAACGAGACGGCCCTCATCATCACCTATGATGAGCATGGTGGCTTCTATGACCATGTT
CCCACGCCTGTCAAAGTGCCTCAGCCTGATGGGATTATTGGCCCTGACCCTTACTACTTC
AAGTTTGATCGGCTCGGGGTGCGCGTGCCCAGCTTCCTCATCTCCCCCTGGGTTGAGAAG
GGCACTGTGATCCATGAACCAAATGGTCCGAAGGAAGACTCACAATATGAGCATTCATCC
ATCCCTGCGACAGTAAAGAGGCTATTTAATTTACGTGCTAACTACCTGACAAAGAGGGAT
TCATGGGCTGGGACCTTTGAGAGCTACCTCAAAGTCCGAAAGACACCAAGAACTGATTGT
CCAGAGAAACTCCCAGAGGTTACAAAGTCCCTGCGACCATTTGGTGCTAATGAAGATAAA
TCTCTATCGGAGTTTCAAGTGGAGTTGATTCAACTTGCCTCTCAGCTCAATGGTGACCAC
GTGCTCAACAGCTATCCGGATATTGGCAAGACGATGAGTGTAGGCGAAGCAAACCGCTAC
GCAGAGGATGCTGTCTCCAGATTCCTGGAGGCTGGAAGGATCGCTCTTAGGGCTGGTGCA
AACGAATCTGCTCTGGTGACAATGAGGCCCGCGCTCACCAGCAGAGCCGCAATGTCCACC
GGCTTGTCATCTGAACTCTGATGGACTGTTACTGGATACTGCCAACAGATTCTGCATCAC
GATTACCGGCGCTACATGGGCTGCAAGAGTACGGTAGCATTTATGTAATGGGTATTAGAT
CTTGCACATGGATTTTTTGTACATATACGCCATGATACTTCGTATCTGAAGCTTACTTCA
GCGGGCCCTCGTGGTTTACGCTCAGGAGTATCACCCAACGACGCACCAGATGCATGCCAA
ATTCCCCTTCCGGATAAGCTGTAGCTGGTGTTGTACCTATTCAGCTCTCTACTGTACCTG

CTTGTATAGCAGTTGAACTGCATCGTAAGGAGTATTGTACTATGAATGATTGTTGGATTT
GGC

4. *PI-PLC2* sequence (cluster 3141-contig 1, assembly sequence in FGAS)

CCACGCGTCCGGTCTCCCTACCCGGTTATCATCACACTCGAAGACCACCTTACACCCGAG
CTGCAGGACAAAGTTGCCAAGATGGTCCTTGAAGTGTTTGGCGACATACTGTACTACCCT
CAAGAAGAACATCCCAAAGAACTCCCTTCACCTGAGTTCCTCAAGGGTCGTGTGCTACTA
TCAACAAAGCCCCCAAAGGAGTACCTTGAAGCCAAGGATGGTGGTGCCGTGAAAGATGGT
GATGCGGAGCAGAATCCTGGCAAAGGGACTGACGATGATGCGGCTTGGGGAACAGAAGTC
CCAGATTTCAAGACTGAAATCCAGTCTGCTAAAGAGGAGGATGCCTCAGAGCACCGTAGA
GATGACGACGAGGACGATGACGATGAGGACGAACAGAAAATGCAACAGCATCTAGCTCCA
CAGTATAAACACCTTATTACTATAAGAGCAGGAAAGCCAAAGGGGGGTACTACGTCTGAT
GCCTTGAAGTGTGACCCAAACAAAGTTAGGCGGCTCAGTTTGAGCGAGCAACAACTTGCC
AAAGCTGTAGTTAATCATGGCACCGAAATAGTGAGGTTTACACAGAGGAATCTTCTGAGG
ATATACCCAAAGGGCACTCGGGTTACTTCATCCAACTATAATCCATTTATTGGTTGGGTG
CACGGTGCTCAGATGGTGGCCTTCAATATGC

5. *PI-PLC3* sequence (cluster 20268-contig 1, assembly sequence in FGAS)

ACCCACGCGTCCGCCGATTTCTACGCCAGGGTGGGGATCGCGGGGGTGCGGGCGGACTGC
GTGATGAAGAAGACCCGGACGATCGAGGACCAGTGGGTGCCGGTGTGGGACGAAGAGTTC
ACGTTCCCGCTGACGGTGCCGGAGCTGGCCCTGCTGCGGGTGGAGGTCCAGGAGTACGAC
ATGTCGGAGAAACACGACTTCGGCGGGCAGACGTGCCTGCCGGTGATGGAGCTGAAGCAG
GGCATCCGCGCCGTGCCCCTCCATGACCGCAAGGGCAACAGGTACAAGTCCGTCAGGCTC
CTCATGCGCTTCGAGCTCGTCTAGCCAAACGGTCGGCATTGTGGAGGTCATATTATGTGG
TGGTAGGAGTAGTCATATTGTCGGAATGATGATAATAATAATGGTAATGTTCTTTGTGTA
GTAGCAAGTCAGGGAGTTGTGTTAAAATTATCTGCTGTGCTAGTTACTGCTGCAGCGGGG
AGGAAAAGTGCAGTGCAGTATATACTGGGGTGGGGCGAGTAAGATTTTTGTGACAGGAAT
AAATGTTTCATTTTGGAGCAAAAAAAAAAAAAAAAAAAAAAA